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Chemotherapy-induced Dkk-1 expression by primary human mesenchymal stem cells is p53 dependent

Ian Hare^{1,2}, Rebecca Evans¹, James Fortney¹, Blake Moses¹, Debbie Piktel¹, William Slone¹, and Laura F. Gibson^{1,2}

¹Alexander B. Osborn Hematopoietic Malignancy and Transplantation Program of the Mary Babb Randolph Cancer Center, Robert C. Byrd Health Sciences Center, West Virginia University School of Medicine, PO Box 9300, Morgantown, WV, USA

²Department of Microbiology, Immunology, and Cell Biology, Robert C. Byrd Health Sciences Center, West Virginia University School of Medicine, PO Box 9104, Morgantown, WV, USA

Abstract

Mesenchymal stem cells (MSCs) are abundant throughout the body and regulate signaling within tumor microenvironments. Wnt signaling is an extrinsically regulated pathway that has been shown to regulate tumorigenesis in many types of cancer. After evaluating a panel of Wnt activating and inhibiting molecules, we show that primary human MSCs increase the expression of Dkk-1, an inhibitor of Wnt signaling, into the extracellular environment following chemotherapy exposure in a p53-dependent manner. Dkk-1 has been shown to promote tumor growth in several models of malignancy, suggesting that MSC-derived Dkk-1 could counteract the intent of cytotoxic chemotherapy, and that pharmacologic inhibition of Dkk-1 in patients receiving chemotherapy treatment for certain malignancies may be warranted.

Keywords

Chemotherapy; Wnt Signaling; Tumor Microenvironment; Dkk-1; Mesenchymal Stem Cell

Introduction

MSCs are a mesenchymal-derived stromal population that is capable of differentiating into osteoblasts, adipocytes, and chondrocytes [3]. Although abundant in bone and adipose tissues, MSCs have been shown to be actively recruited specifically to sites of the body where they regulate angiogenesis, inflammation, and invasion of tumor cells [4]. Once within this functionally distinct tumor niche, MSCs influence the behavior of a tumor through the expression of various proteins which regulate signaling pathways that influence survival and proliferation [5]. The Wnt signaling pathway has been described as influencing

Correspondence to: Ian Hare.

Compliance with ethical standards

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malignancy in certain tumor microenvironments, extrinsically regulated by the abundance of secreted activating and inhibitory molecules into the extracellular milieu [6]. Humans express 19 Wnt activating ligands, and several inhibitory proteins including soluble Frizzled proteins (SFRP) and Dickkopf proteins (Dkk), which inhibit Wnt signaling by quenching extracellular Wnt ligands and preventing the assembly of the Wnt surface receptor complex, respectively [7]. MSC-derived Wnt ligands have been shown to initiate an epithelial-mesenchymal transition phenotype of colon cancer cells [8], and activation of Wnt signaling with lithium chloride reduced tumor burden in a murine model of myeloma [9], highlighting the importance of Wnt signaling in the tumor microenvironment.

We have reported previously that stromal cells of the bone marrow have a reduced ability to support optimal hematopoietic cell function following exposure to chemotherapy [2, 10, 11]. Given the observation that MSCs express Wnt activating and inhibiting molecules [12], and the importance of Wnt signaling within the tumor microenvironment, we evaluated the expression of Wnt-regulating molecules by primary human MSCs following chemotherapy exposure. In the current study, we show that Dkk-1, an inhibitor of Wnt signaling that has been implicated in the progression of various tumors, is elevated in primary human MSCs after exposure to various chemotherapeutic agents.

Materials and methods

MSC acquisition and cell culture

MSCs cultures were derived from de-identified bone marrow specimens from patients at the West Virginia University Cancer Institute and cultured as previously described [1]. Patients had no history of exposure to chemotherapy, radiation, or malignancy. HS-5 and HS-27A cell lines were purchased from ATCC (Manassas, VA, USA) and cultured as suggested by the supplier.

Chemotherapy and drug exposures

Chemotherapy exposure was at sublethal concentrations for all experiments. Etoposide (VP16) and melphalan were prepared as previously described [2], and 5-FU (Selleck Chemicals, Boston, MA, USA) was diluted to 10 µg/mL in DMSO prior to use. GolgiStop (BD Biosciences, San Jose, CA, USA) was used following manufacturer's guidelines. Pifithrin-α, nutlin-3, and RU-486 (Selleck Chemicals, Boston, MA, USA) were diluted in DMSO, and recombinant

Wnt3a (R&D Systems, Minneapolis, MN, USA) was dissolved in phosphate buffered saline prior to use.

RNA isolation and qPCR

RNA was isolated and analyzed by qPCR as previously described [1]. The following primer sequences were used (5'–3'), DKK1 (F-CGTCACGCTATGTGCTGCC, R-GCTTTCAGTGATGGTTTCCTCA), SFRP1 (F-AGTTCTTCGGCTTCTACTGGC, R-AACTCGTTGTACAGGGAGG AC), WNT2B (F-TTGACAACTCTCCAGATTACTGTGT, R-ATTTACAACCGTCTGTTTCCTT), WNT3A

(F-GATGGTGTCTCGGGAGTTTCG, R-GTGGCACTTGCACTTGAGGT), WNT4 (F-GGTCACGCACTGAAGGAGAAG, R-CAAGTACACCAGGTCCTCATCTGT), WNT5A (F-CTCTGTTTTTGGCAGGGTGA, R-GCAGCCGCAGGTGGACA), WNT10B (F-GGTCCACGAGTGTCAGCAC, R-CAGCCAGCATGGAGAAGGA), and GUSB (F-AAACGATTGCAGGGTTTCAC, R-CTCTCGTCGGTGACTGTTCA).

Western blotting

Western blotting was performed as previously described [1], using the following antibodies from Cell Signaling Technologies (Danvers, MA, USA), Dkk-1 (#4687), p53 (#2524), and Phosphorylated-p53 (#9286). Antibodies were diluted as directed by manufacturer.

Dkk-1 ELISA

Dkk-1 ELISA was performed using Human DuoSet Dkk-1 ELISA (R&D Systems, Minneapolis, MN, USA) following manufacturer's recommendations. Cellular supernatants were diluted 1:4 prior to analysis.

p53 siRNA Knockdown

p53-specific siRNA and scramble controls were utilized, obtained from GE Dharmacon (Lafayette, CO, USA), and used following manufacturer's protocol. Knockdowns were performed overnight, followed by 48-h recovery in basal culture medium prior to chemotherapy exposure.

Results

Dkk-1 expression is elevated in primary human MSCs after chemotherapy exposure

To determine whether chemotherapy exposure alters the expression of MSC-derived Wnt-regulating molecules, MSCs were exposed to etoposide (VP16) for 24 h followed by qPCR analysis. Of a panel of Wnt activating and inhibitory molecules, VP16 significantly increased the abundance of DKK1 mRNA (Fig. 1a). DKK1 transcripts were elevated following exposure to VP16, melphalan, and fluorouracil (5-FU), indicating that the response was consistent among chemotherapeutics with various mechanisms of action (Fig. 1b). Dkk-1 protein was also elevated, evaluated by Western blot analysis (Fig. 1c), following inhibition of secretion using GolgiStop (BD Biosciences). The requirement for inhibition of secretion for detectable protein accumulation suggests that Dkk-1 is quickly secreted following transcription (Fig. 1c). Finally, VP16, melphalan, and 5-FU elevated the abundance of Dkk-1 protein in culture supernatants (Fig. 1d). These results indicate that primary human MSCs display elevated Dkk-1 expression following exposure to cytotoxic stress by drugs with various mechanisms of action.

Elevated Dkk-1 expression is regulated by p53

The necessity of GolgiStop to visualize intracellular Dkk-1 by Western (Fig. 1c) suggested that the protein is rapidly secreted and that elevated expression of Dkk-1 following chemotherapy exposure was potentially regulated at the mRNA level. To determine the mechanism by which Dkk-1 is elevated following chemotherapy-induced stress, we

investigated the potential influence of signaling pathways with promoting elements within the DKK1 promoter. The promoter region of DKK1 has been shown to contain several TCF/LEF response elements, enabling β -catenin-mediated gene transcription following activation of the Wnt signaling pathway [13]. Given that exogenous activation of Wnt signaling by rWnt3a does not increase the transcriptional abundance of DKK1, and the nuclear abundance of β -catenin is not increased following VP16 exposure (Supplemental Fig. 1), it is unlikely that Wnt signaling regulates DKK1 expression in MSCs following chemotherapy exposure. Glucocorticoid response elements (GRE) are also present within the DKK1 promoter [14], consistent with the elevation of DKK1 mRNA following dexamethasone exposure (Supplemental Fig. 2a). However, inhibition of GRE signaling using RU-486 does not diminish chemotherapy-induced DKK1 elevations following chemotherapy exposure (Supplemental Fig. 2b), suggesting that glucocorticoid signaling does not regulate DKK1 in MSCs after chemotherapy.

Previously, the promoter region of DKK1 was shown to contain a p53 response element [15]. Exposure of MSCs to VP16, melphalan, or 5-FU resulted in increased levels of phosphorylated p53 (Fig. 2a). Inhibition of the transcriptional function of p53 with pifithrin- α resulted in a dose-dependent decrease in DKK1 abundance when combined with melphalan exposure (Fig. 2b). In addition, inhibition of p53 by targeted siRNA resulted in decreased abundance of soluble Dkk-1 from control, as well as chemotherapy exposed, MSCs (Fig. 2c). Conversely, activation of p53 using nutlin-3, an MDM2 inhibitor, increased DKK1 abundance in a dose-dependent manner when combined with melphalan (Fig. 2d). Contrary to primary MSCs, HS-5 and HS-27A human cell lines display deregulated p53 function due to the expression of human papillomavirus virus E6 and E7 proteins [16]. Consistent with the necessity of p53 to elevate Dkk-1 expression following chemotherapy in primary MSCs, exposure of HS-5 and HS-27A cells to sublethal concentrations of chemotherapy or nutlin-3 did not elevate Dkk-1 expression (Supplemental Fig. 3), unlike primary human MSCs (Figs. 1d, 2d). These results suggest that p53 regulates the elevated expression of Dkk-1 following exposure of MSCs to various chemotherapeutics.

Discussion

Our results show that MSC-derived Dkk-1 is elevated in cells following cytotoxic drug exposure suggesting that it could be elevated in the tumor microenvironment following treatment with chemotherapy. VP16, a topoisomerase II inhibitor, melphalan, a DNA alkylating agent, and 5-FU, a pyrimidine analog that inhibits thymidine synthase [17–19], were all capable of increasing Dkk-1 expression, indicating the broad spectrum of stressors which can elicit such an effect. Elevated Dkk-1 could have negative consequences for patients suffering from certain tumors. For example, Dkk-1 has been shown to promote the migration and invasion of hepatocellular carcinoma cells [20], and to promote the growth of certain esophageal, non-small cell lung cancers, and myeloma cells [21–23]. The effects of Dkk-1 appear tumor specific, since Dkk-1 has been shown to act as a tumor suppressor in certain cancers, such as melanoma and colon [24, 25]. Nevertheless, these findings suggest that chemotherapy could be having an undesired effect in these cancers, potentially leading to unsuccessful treatment or relapse of disease.

To our knowledge, this is the first time Dkk-1 has been shown to be elevated in MSCs following exposure to chemotherapy. However, it has been shown previously that certain cancer cell lines with functional p53 react similarly in response to chemotherapy-induced stress [26]. Importantly, our observations are the first to include primary human MSCs which have been shown to be a component of the tumor microenvironment. Wang et al. and Shou et al. [15, 27] have also described the role of p53 in chemotherapy-induced Dkk-1 expression, supporting and refuting the notion in tumor cell lines, respectively. Our data are consistent with the assertion that p53 is playing a role in this regulation; however, it is likely that the phenomenon is regulated differently between cell types. The necessity of functional p53 for normal cellular function makes targeting the p53 pathway unfavorable in conjunction with cytotoxic therapies, suggesting that pharmacologic targeting of this response would be best directed at soluble Dkk-1 protein.

These observations align with a recent publication describing increased Dkk-1 protein in the serum of patients who have received chemotherapy [28], indicating that MSCs could be one potential source of this response. BHQ-880, a monoclonal anti-Dkk-1 antibody which has cleared phase Ib clinical trials [29], could theoretically be used in conjunction with chemotherapy to prevent the undesired effects of Dkk-1 on tumor phenotype in patients being treated with chemotherapy for certain malignancies. A more in depth analysis needs to be performed to understand which tumors respond to Dkk-1 as an oncoprotein and which respond to Dkk-1 as a tumor suppressor prior to use in vivo. Overall, our findings show that chemotherapy treatment may have an undesirable effect on the tumor microenvironment with future work required to evaluate whether MSC-derived Dkk-1 negatively influences tumor behavior in the context of certain malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Dkk-1 is elevated in MSCs following chemotherapy exposure. **a** qPCR evaluating Wnt-inhibitory and activating molecules in MSCs exposed to 100 μ M etoposide (VP16) or diluent control for 24 h. **b** qPCR evaluating DKK1 expression in MSCs exposed to 100 μ M VP16, 25 μ g/mL melphalan (Mel), 300 μ g/mL fluorouracil (5-FU), or medium-only control. VP16 and melphalan exposures were 24 h, 5-FU was incubated with MSCs for 1 h followed by 23-h incubation in normal growth medium. These chemotherapy conditions were kept consistent throughout the remaining experiments unless otherwise specified. **c** Western blot analysis of Dkk-1 in MSCs exposed to chemotherapy or medium-only control, in the presence of GolgiStop or diluent alone for 24 h. **d** Dkk-1 ELISA analysis of supernatants from MSCs exposed to chemotherapy or medium-only control for 72 h. 5-FU was administered for 1 h followed by 72-h incubation in basal medium. *Indicates p value < 0.05 , using Student's T test (*a*) or One-way ANOVA with Dunnett's post hoc (*b, d*)

Fig. 2.

p53 regulates Dkk-1 following chemotherapy exposure of MSCs. **a** Western blot analysis of phosphorylated (phospho) and total p53 protein in MSCs following 6 h exposure to chemotherapy or medium-only control. **b** qPCR evaluation of DKK1 in MSCs exposed to melphalan in combination with increasing concentrations of pifithrin- α (PTH α) for 24 h. Cells were pre-treated with PTH α for 20 min prior to chemotherapy, and PTH α was spiked into culture vessels every 8 h. **c** Dkk-1 ELISA analysis of MSCs transfected with two separate siRNA targeting p53 mRNA (or scramble control) and then exposed to chemotherapy or medium-only control for 24 h. Cells conditioned medium for 72 h prior to analysis. **d** qPCR evaluation of DKK1 in MSCs exposed to melphalan in combination with increasing concentrations of nutlin-3 for 24 h. Cells were pre-treated with nutlin-3 for 20 min prior to chemotherapy. *Indicates p value <0.05, Oneway ANOVA with Holm-Sidak post hoc