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Research Paper

Chemotherapy and Stem Cell Transplantation Increase *p16*^{INK4a} Expression, a Biomarker of T-cell Aging

William A. Wood [a], Janakiraman Krishnamurthy [a,b], Natalia Mitin [a,b], Chad Torrice [a,b], Joel S. Parker [b], Anna C. Snavely [a], Thomas C. Shea [a], Jonathan S. Serody [a], Norman E. Sharpless [a,b,*]

[a] Department of Medicine, The Lineberger Comprehensive Cancer Center, The University of North Carolina School of Medicine, Chapel Hill, NC, USA
[b] Department of Genetics, The Lineberger Comprehensive Cancer Center, The University of North Carolina School of Medicine, Chapel Hill, NC, USA

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ABSTRACT

The expression of markers of cellular senescence increases exponentially in multiple tissues with aging. Age-related physiological changes may contribute to adverse outcomes in cancer survivors. To investigate the impact of high dose chemotherapy and stem cell transplantation on senescence markers in vivo, we collected blood and clinical data from a cohort of 63 patients undergoing hematopoietic cell transplantation. The expression of *p16*^{INK4a}, a well-established senescence marker, was determined in T-cells before and 6 months after transplant. RNA sequencing was performed on paired samples from 8 patients pre- and post-cancer therapy. In patients undergoing allogeneic transplant, higher pre-transplant *p16*^{INK4a} expression was associated with a greater number of prior cycles of chemotherapy received ($p = 0.003$), prior autologous transplantation ($p = 0.01$) and prior exposure to alkylating agents ($p = 0.01$). Transplantation was associated with a marked increase in *p16*^{INK4a} expression 6 months following transplantation. Patients receiving autologous transplant experienced a larger increase in *p16*^{INK4a} expression (3.1-fold increase, $p = 0.002$) than allogeneic transplant recipients (1.9-fold increase, $p = 0.0004$). RNA sequencing of T-cells pre- and post- autologous transplant or cytotoxic chemotherapy demonstrated increased expression of transcripts associated with cellular senescence and physiological aging. Cytotoxic chemotherapy, especially alkylating agents, and stem cell transplantation strongly accelerate expression of a biomarker of molecular aging in T-cells.

1. Introduction

Hematopoietic stem cell transplantation (HSCT) is a potentially curative modality for high-risk hematologic diseases, but the procedure has profound and long-term effects on recipient hematologic and immune function. The long-term toxicity of HSCT may result from chemo-radiotherapy given at the time of transplantation (conditioning), from donor-host immune differences after allogeneic transplants or from accelerated stem cell exhaustion of transplanted stem cells (Hake et al., 2007). These late toxicities manifest as increased risk for infection, chronic graft-vs-host disease, bone marrow failure and acute leukemia.

Recent evidence has demonstrated that peripheral blood T-cells express markers of cellular senescence with physiological aging. The overall loss of physiological reserve that accompanies aging is associated with an accumulation of senescent cells (Sharpless and DePinho, 2007; Rodier and Campisi, 2011). Cellular senescence is triggered by activation of tumor suppressor mechanisms associated with cellular stressors, and results in expression of the *p16*^{INK4a} tumor suppressor protein encoded by the *CDKN2a* locus, which has emerged as one of the more useful markers of senescence in vivo (Campisi, 2013; Sharpless and Sherr, 2015). Expression of *p16*^{INK4a} in peripheral blood T lymphocytes increases exponentially with chronological age, doubling about every decade (Zindy et al., 1997; Krishnamurthy et al., 2004; Liu et al., 2009). Polymorphisms of senescence regulators have been associated with age-related conditions such as cancer, pulmonary fibrosis, glaucoma, atherosclerosis, and type II diabetes (Jeck et al., 2012; Siegel et al., 2012). Prior work has shown that several age-promoting stressors such as smoking, physical inactivity and chronic HIV infection accelerate the expression of *p16*^{INK4a} and other markers of cellular senescence (Liu et al., 2009; Nelson et al., 2012). Importantly, we recently showed that cytotoxic chemotherapy, given in the adjuvant setting, markedly increases expression of senescence markers in the peripheral blood, consistent with ~15 years of chronological aging (Sanoff et al., 2014).

Increasingly, older individuals are considered for autologous or allogeneic transplantation. While age itself is usually not considered an absolute contraindication to transplantation, older individuals do have

* Corresponding author at: The Lineberger Comprehensive Cancer Center, CB #7295, Departments of Medicine and Genetics, The University of North Carolina School of Medicine, Chapel Hill, NC 27599-7295, USA.
E-mail address: NES@med.unc.edu (N.E. Sharpless).

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higher risks of acute transplant-related toxicities such as cardiac arrhythmias, diarrhea and mucositis (Wildes et al., 2014). Further, age-related comorbid illness is itself prognostic for outcomes in autologous and allogeneic transplant recipients, suggesting that functional, if not chronological, age of prospective transplant candidates is a potentially important variable for clinical decision-making. Lastly, survivors of transplants, regardless of age, are at risk for accelerated acquisition of several age-related syndromes such as endocrine dysfunction, cognitive impairment, cardiovascular morbidity, immune dysfunction, secondary neoplasms, and neuromuscular impairment (Fried et al., 2001).

In murine models, serial transplantation per se, in the absence of exposure to cytotoxic agents, is associated with accelerated aging of hematopoietic stem cells (HSC), manifesting as 'HSC exhaustion' (Harrison and Astle, 1982). Likewise, evidence suggests HSC exhaustion occurs in humans as well. HSC yields for autologous transplant from patients that have undergone significant prior chemotherapy are significantly depressed compared to yields from less heavily treated individuals (Clark and Brammer, 1998), and the transplantation of insufficient numbers of HSC is associated with long term graft failure (Perez-Simon et al., 1999). Additionally, transplantation is associated with an increased rate of telomere shortening, which has been associated with certain adverse outcomes in transplant recipients (Lee et al., 1999; Lewis et al., 2004; Akiyama et al., 2000; Pipes et al., 2006). Because individuals with hematologic malignancies have an increasing array of transplant approaches of varying intensity as well as non-transplant treatment approaches available to them, understanding the impact of treatment upon functional aging may have important implications for the care of both prospective transplant candidates as well as transplant survivors. Toward that end, we measured expression of *p16^{INK4a}*, a marker of molecular age that can be serially assessed, in HSC-derived T-cells before and after stem cell transplantation. Additionally, we performed whole transcriptomic RNA sequencing in a subset of paired samples to further examine the effects of chemotherapy or transplantation on T-cell function.

2. Materials and Methods

2.1. Patients

For the transplant patient population, participants were over the age of 18 and underwent either autologous or allogeneic stem cell transplantation for any hematologic malignancy between 2010 and 2013 at the University of North Carolina (UNC) Hospitals. Patient samples were obtained from two non-randomized, non-blinded observational cohorts: a study investigating symptom burden after transplantation, and a generic tissue procurement protocol. Studies were approved by the UNC Institutional Review Board (11–0600 and 13–1705), with study procedures confirming to standards indicated by the Declaration of Helsinki. Eligible patients were identified from the electronic medical records and approached by research personnel prior to scheduled transplantation for provision of signed informed consent. Patients undergoing concurrent radiation, chemotherapeutic, or investigational therapy other than transplant-related therapy were excluded. All patients received standard-of-care therapies and treatments as clinically needed. Medical history and treatment information were abstracted from the medical record. Samples were obtained in both cohorts from just before transplantation, and paired samples from 6 months post-transplantation were also obtained if available. Molecular analyses were performed by investigators blinded to patient data, and investigators collecting clinical information were blinded to laboratory results until data collection was complete. For the breast cancer patient population, T-cell RNA collected in the study Sanoff et al. (Sanoff et al., 2014) was used in the RNA sequencing analysis.

2.2. Assessment of *p16^{INK4a}* expression

See Sanoff et al. (Sanoff et al., 2014) for details. In brief, CD3$^+$ T-cells were isolated from up to 10-ml of peripheral blood using anti-CD3 microbeads and an AutoMACS^{PRO} separator (Miltenyi Biotec, San Diego, CA). Purity of T cells was determined to be ~95% when isolated from fresh blood and ~50% when isolated from cryopreserved PBMCs in pilot experiments. T cell purity in clinical trial samples was monitored by measuring expression of the gamma subunit of the *CD3*. Total RNA was isolated using RNeasy Mini Kit (Qiagen) and cDNA were prepared using ImProm-II reverse transcriptase kit (Promega). Expression of *p16^{INK4a}* was measured by TaqMan quantitative reverse-transcription polymerase chain reaction specific for *p16^{INK4a}* and normalized to *YWHAZ* housekeeping gene (Mane et al., 2008; Dheda et al., 2004).

2.3. RNA Sequencing

RNA was extracted and rRNA was removed using the Ribo-Zero kit. RNA libraries were prepared by using the Illumina TruSeq RNA Sample Preparation Kit v2 and then sequenced by Illumina HiSeq2000. Reads were subjected to quality control as previously described (Cancer Genome Atlas Research, 2012). RNA reads were aligned to human hg19 genome assembly using Mapsplice (Wang et al., 2010). Gene definitions were obtained from the UCSC known Gene table. Gene expression was estimated using RSEM (RNA-Seq by Expectation Maximization) (Li and Dewey, 2011). Genes differentially expressed due to treatment were identified by DESeq2 (Love et al., 2014) using a bivariate model to adjust for subject specific effects. The resulting statistics were subjected to gene set enrichment analysis by using the GSEA (Gene Set Enrichment Analysis) rank test (Subramanian et al., 2005). Expression estimates were normalized to a fixed upper quartile and log2 transformed prior to visualization.

2.4. Statistical Analyses

The sample size was determined by the availability of clinical specimens from the two study cohorts as described. Log_2-transformed *p16^{INK4a}* expression values were standardized through conversion to *Z*-score to facilitate combining the two sample sets. Z-scores were calculated separately for the two transplant cohorts using the formula:

$$Z_i = (X_i - \mu)/\sigma$$

where μ is population mean, and σ is standard deviation.

For samples present in both cohorts, individual Z scores were averaged. Associations between *p16^{INK4a}* expression and pre-transplant variables were performed using linear regression (for continuous variables) or one-way analysis of variance (for categorical variables). A paired *t*-test was used to compare *p16^{INK4a}* expression before and after transplant. Data were analyzed by N. Mitin using JMP11 (SAS, Cary, NC) and A. Snavely using R. All tests of statistical significance were two-sided. *P* values of 0.05 or less were considered statistically significant.

3. Results

Two observational cohorts (Table 1) were combined for our analysis, and the baseline characteristics of the aggregated cohort are shown in Table 2. In order to compare samples analyzed from the two observational cohorts, we converted all *p16^{INK4a}* expression values to a normalized *Z*-score as described in the methods. Using this conversion, we found excellent correlation among Z-scores for the 17 patients that had separate samples obtained in both cohorts (Table 3), suggesting the aggregation of the observational cohorts for analysis is valid. In the combined cohort there were 26 unique patients who underwent autologous transplantation and 37 who underwent allogeneic

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