H2-Ea Deficiency Is a Risk Factor for Bleomycin-Induced Lung Fibrosis in Mice

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Abstract

Pulmonary fibrosis, an incurable late-stage lung injury with a high mortality, is a significant complication in cancer patients when treated by radiation, e.g., thoracic malignant diseases, or chemotherapeutic agents. Bleomycin is one of the primary drugs used to treat testicular cancer, but the incidence of significant pulmonary fibrosis limits the dose. It is known that susceptibility to bleomycin-induced pulmonary fibrosis is a heritable trait controlled by multiple genes, none of which, however, are yet known. In this study, we used expression profiling and genetic analysis in mouse models of bleomycin-induced pulmonary fibrosis and identified MHC class II antigen Ee (H2-Ea) as a risk factor for this disease. We found that a loss-of-function deletion in the H2-Ea gene was linked to susceptibility. A functional test of H2-Ea in transgenic mice showed 100% survival in the transgenic mice compared with 53% in C57BL/10J mice and significantly decreased pulmonary fibrosis from 16.42% (C57BL/10J) to 5.76% (transgenic; P = 1.20e^{-6}). These results show that H2-Ea expression protects mice from bleomycin-induced pulmonary fibrosis, which implicates H2-Ea as a candidate susceptibility gene for pulmonary fibrosis. It is known that susceptibility to bleomycin-induced pulmonary fibrosis is a heritable trait controlled by multiple genes, none of which, however, are yet known. In this study, we used expression profiling and genetic analysis in mouse models of bleomycin-induced pulmonary fibrosis and identified MHC class II antigen Ee (H2-Ea) as a risk factor for this disease. We found that a loss-of-function deletion in the H2-Ea gene was linked to susceptibility. A functional test of H2-Ea in transgenic mice showed 100% survival in the transgenic mice compared with 53% in C57BL/10J mice and significantly decreased pulmonary fibrosis from 16.42% (C57BL/10J) to 5.76% (transgenic; P = 1.20e^{-6}). These results show that H2-Ea expression protects mice from bleomycin-induced pulmonary fibrosis, which implicates H2-Ea as a candidate susceptibility gene for pulmonary fibrosis.

Introduction

Pulmonary fibrosis, an incurable late-stage lung injury with a high mortality, is a significant complication in cancer patients when treated by radiation, e.g., thoracic malignant diseases, or chemotherapeutic agents (1). Bleomycin is one of the primary drugs used in the chemotherapeutic regimen for testicular cancer, but it also carries a high risk of inducing pulmonary fibrosis. About 10% of patients treated with bleomycin develop pulmonary fibrosis, and the incidence of death is as high as 2%. In children, the incidence may be even higher (1). Thus pulmonary toxicity is a serious dose-limiting complication in the treatment of cancer with bleomycin.

Studies of bleomycin-induced lung fibrosis in mouse models indicate that susceptibility is genetically regulated, at least in part. Genetic mapping has been used to locate susceptibility loci on chromosomes 17, 11, 6, and 13 with mouse models (2, 3). The chromosome 17 locus, named Blmpf1, is within the major histocompatibility complex (MHC; ref 2). The importance of this locus is underscored by the association of the MHC haplotype with susceptibility to pulmonary fibrosis in both humans and experimental animal models (2, 4, 5). Moreover, linkage loci for pulmonary fibrosis after other insults such as radiation, particulates, or ozone exposure all mapped to the same region (6–8), which suggests that this may be a common fibrosis locus. However, genes underlying Blmpf1 remain unknown.

It is well documented that cytokines and growth factors are involved in lung injury and the development of pulmonary fibrosis, including tumor necrosis factor-α, transforming growth factor-β, and interleukins (9). However, the regulation of pulmonary fibrosis by these genes and their relationship with Blmpf1 are not known.

The purpose of our study was to identify genes underlying Blmpf1 and to better understand the pathways involved in the regulation of bleomycin-induced pulmonary fibrosis. We compared gene expression profiles between fibrosis-resistant strain C3H and its fibrosis-prone congenic strain by with DNA microarrays. We demonstrate that H2-Ea, a MHC class II antigen gene, is a candidate susceptibility gene underlying Blmpf1.

Materials and Methods

Mice. All of the mouse breeding and experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center. Inbred C3Hf/Kam (C3) and C57BL/6J (B6) mice were bred and maintained in the specific pathogen-free animal colony of the Department of Experimental Radiation Oncology. All of the other inbred and congenic strains of mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in the specific pathogen-free animal colony of the Department of Veterinary Medicine. H2-Ea transgenic B10.Ea8 mice were the generous gift of Dr. Chella David of Mayo Clinic, Rochester, MN. The H2-Ea (k) gene from A/J strain was originally microinjected into (B6 × SJL) F2 embryos to generate the original transgenic line, which was backcrossed to C57BL/10J (B10) for 12 generations to generate the B10.Ea8 transgenic line.

Bleomycin Treatment. We treated 8-week-old male mice with 100 mg/kg bleomycin (Bristol-Myers Squibb, Evansville, IN) via 7-day osmotic minipumps (ALZA Corp., Palo Alto, CA) to elicit pulmonary damage, as described previously (10). This systemic administration of bleomycin induced a nonpulmonary systemic toxicity from 7 to 21 days after pump implantation, followed by a lung damage phase from 21 to 56 days, during which some mice exhibited symptoms of lung damage, i.e., increased breathing rate and dramatic loss of weight, necessitating sacrifice, as described previously (10). All of the surviving mice were sacrificed at 56 days. For the microarray study, we sacrificed three mice from each strain at 0, 7, 14, 21, 35, and 56 days after bleomycin treatment.

Histology and Fibrosis Scoring. At euthanasia, we removed the left lobe of the lungs and perfused the lobes with 10% neutral buffered formalin in preparation for histologic examination. Sections were stained with H&E and Masson’s Trichrome to identify fibrosis in the lung and were scanned with a video imaging system, as described previously (10), to obtain images, which were blindly scored separately by two individuals (M. D. and E. L. T.) to quantify fibrosis (reported as the ratio of fibrotic lung area to total lung area), with the assistance of computer software. Specifically, the area of fibrosis in the left lung lobe was determined from a user-drawn region surrounding the fibrosis and was compared with the area of the entire lobe to yield the percentage of pulmonary fibrosis (%PF) for individual mice (10).

Labeling of Target cDNA and Hybridization to Microarrays. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) from the right lobes of lungs from each mouse. cDNA was radioactively labeled to hybridize a mouse microarray containing 1,176 cancer-related genes on a nylon membrane (BD Biosciences, San Jose, CA). We used at least three mice for each time point.

Microarray Scanning. After hybridization, each array was washed and then exposed to a storage phosphor screen for image scanning on a Storm scanner (Molecular Dynamics, Sunnyvale, CA).
Data Analysis. The signal intensities of each array image were quantified with ArrayVision (Imaging Research, St. Catharines, Ontario, Canada). Background was subtracted, and expression levels were normalized within each array against the 75th percentile of the expression level of all genes. Genes with a signal-to-noise ratio of ≤2 were excluded from further analysis. The remaining data were analyzed with SPLUS (Insightful, Corp, Seattle, WA) “gam” procedure for fitting generalized additive models. The object was to determine whether expression profiles differed between the two strains over time. Two models were fitted. The first model fitted a smooth curve to expression levels without considering any strain effect, and the second fitted a smooth curve to the different strains separately. An ANOVA was performed on the residuals, which evaluated whether or not the more complex model (modeling differences between strains) explained a statistically significant amount of variation, compared with the simpler model (no difference between strains). We used $P = 0.05$ as a cutoff to select candidate genes for different expression profiles between the two strains: C3H and C3.SW-H2b.

**H2-Ea Genotyping.** Genomic DNA from mice was used for PCR analysis of H2-Ea gene polymorphisms. The oligonucleotide primers flanking a deletion region used for H2-Ea genotyping were described as below: 5’-GATCCACTGCAAAGGAG-3’ and 5’-GGTTGGTCTCCTACTGAAAGC-3’. The conditions used were as follows: 1 cycle of 94°C for 5 minutes; 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and 1 cycle of 72°C for 10 minutes. PCR products were fractionated in a 1% agarose gel and were visualized with ethidium bromide staining.

**Statistical Analysis.** The statistical package Splus Version 6.1 (Insightful Corp, Seattle, WA) was used for data analysis. The standard Splus linear modeling functions were used to fit and explore a variety of different models to the response variable (percentage pulmonary involvement of fibrosis) as a function of various factors and covariates. As applied here, a linear model is essentially an ANOVA, except that the mean effect of each level of the factors is explicitly estimated. The most general model considered was $\text{Pct-Fib} = \beta_{\text{Strain}} + \beta_{\text{Experiment}} + \epsilon$, where $\text{Pct-Fib}$ is %PF and $\beta_{\text{Strain}}$ and $\beta_{\text{Experiment}}$ are the mean effects of Strain and Experiment (i.e., the different mouse strains and the date of the inception of the experiment).

**Quantitative Real-time Reverse Transcription-PCR.** Quantitative real-time reverse transcription-PCR was used to validate the differential expression of H2-Ea identified by microarray. Total RNA from each mouse at each time point was pooled and treated with DNase I before reverse transcription with SuperScript II RNase H transcriptase and oligo-dT (Invitrogen, Carlsbad, CA). Real-time PCR was carried out with a Rotor-Gene 2000 thermal cycler (Corbett Research, Sydney, Australia) in a volume of 10 µL, which contained 5 µL of SYBR Green JumpStart Taq Mix (Sigma-Aldrich, St. Louis, MO). The final concentration of each oligonucleotide primer, and first-strand cDNA corresponding to 25 ng of total RNA. Cycling conditions were as follows: 2 minutes at 94°C, 40 cycles of 15 seconds at 94°C, 1 minute at 60°C, and 1 minute at 72°C. Fluorescence was measured and acquired at the 72°C step. All of the PCR reactions were run in triplicate. Primers used for H2-Ea were 5’-GGCCATGAGATTTTCCATGT-3’ and 5’-TCACAGGGCTTCTGGAGATG-3’, and 5’-AGCCATGTAAGGCTACCC-3’ and 5’-CTCT-CAGGTGTTGTTGAA-3’ for β-actin. The expression of H2-Ea RNA was normalized to the expression of β-actin.

**Northern Blot Analysis.** Ten micrograms of total RNA were loaded and fractionated in a 1% formaldehyde-containing denaturing agarose gel, and then were transferred to a nylon membrane with a downward transfer method. DNA probe for hybridization was labeled by random-primed labeling with [α-32P]dATP. After hybridization and washing, the membrane was exposed to a storage phosphor screen for image scanning on a Storm scanner (Molecular Dynamics). β-actin was used as control.

**Results**

The congenic strain C3.SW-H2b (congenic C3), which differs from the C3 strain by only 6 to 8 eM within the MHC region on chromosome 17, is susceptible to bleomycin-induced pulmonary fibrosis (2). We analyzed expression profiles between this strain and the resistant C3. By fitting a statistical model to the expression level of each gene over time, we identified 70 differentially expressed genes with $P = 0.05$ as a threshold. Among these genes, H2-Ea had the smallest $P$ value ($1.7 \times 10^{-5}$) and was localized to the interval of Blmpf1 with 1 Mb of the peak linkage marker, D17Mit16. H2-Ea encodes the α chain of MHC class II antigen E, one of two class II antigens that are expressed on the cell surface of antigen-presenting cells including macrophages, B cells, and dendritic cells. They bind and present antigens to T cells, inducing T-cell–mediated immune responses. Lung is one organ in which the surface expression of MHC class II molecules is detected.

In nontreated mice, H2-Ea expression was twice as high in fibrosis-resistant C3 as in fibrosis-prone congenic C3 mice (Fig. 1A). It significantly decreased ($P < 0.05$) in C3 mice by 14 days after bleomycin treatment but returned to pretreatment values after 21 days. In congenic C3 mice, however, H2-Ea expression remained at the same low level postbleomycin. We used quantitative real-time reverse transcription-PCR to verify the differential expression of H2-Ea. The results were similar to the microarray data: H2-Ea expression was detected only in fibrosis-resistant C3 but not in fibrosis-prone congenic C3 or B6, our standard fibrosis-prone mouse (Fig. 1B). Real-time PCR also showed a similar time-dependent expression pattern in C3 mice compared with microarray data. Northern blots showed similar results (Fig. 1C).

The differential expression of H2-Ea is caused by a loss-of-function deletion of ~600 bp in the promoter and first exon of the H2-Ea gene. **Fig. 1. Differential expression of H2-Ea between fibrosis-resistant and -prone mouse strains.** A, microarray analysis of H2-Ea expression in the lungs from fibrosis-resistant C3Hf/Kam (○) and fibrosis-prone C3.SW-H2b (●) mice. B, real-time PCR validation of microarray data of H2-Ea expression in the lungs from fibrosis-resistant C3Hf/Kam (○) and fibrosis-prone C3.SW-H2b (●) and C57BL/6J (●) mice. All of the PCR reactions were run in triplicate. Means and SEM are shown. C, Northern blot analysis. Intensity of each H2-Ea band in C3Hf/Kam mice was quantified and normalized against actin; the results are shown under the gel with the ratio in the C3Hf/Kam mouse at day 0 set at 1.
that occurs in strains of the H2b but not in H2k or H2d haplotype (11). We quantified %PF in 355 bleomycin-treated (C3 × B6) F2 mice with a video-imaging system (10). The %PF for male mice homozygous for the deletion (H2-Ea−/−) was 4.93%, significantly higher than that for H2-Ea+/− and H2-Ea+/+ mice, 1.61% (P < 0.01) and 0.08% (P < 10−5), respectively, showing a strong correlation between H2-Ea genotype and %PF (Fig. 2A). Similar results were observed in female F2 mice (Fig. 2B). The distribution of individual mice by %PF showed that more than 90% of male and female H2-Ea−/− mice had a %PF < 0.5% and none had a %PF > 2.5%, whereas more than 25% of H2-Ea+/− mice had a %PF > 5%, indicating that individuals lacking functional H2-Ea are at a high risk of fibrosis (Fig. 2C). Fig. 2D shows that both the incidence and severity of fibrosis in mice categorized as high (%PF ≥ 3), low (0 < %PF < 3), or nonresponders (%PF = 0) are associated with the H2-Ea genotype.

We used H2-Ea transgenic B10 mice (B10.Ea) as a functional test on April 14, 2017. © 2004 American Association for Cancer Research.
of H2-Ea on bleomycin-induced pulmonary fibrosis. In two separate experiments, overall survival rate was 100% and 53% in the transgenic and B10 mice, respectively (P = 0.0004; Fig. 3A). We quantified fibrosis in moribund B10 mice that were sacrificed during the experiment and in all surviving mice at experiment termination (Fig. 3B and C). We found a strong negative correlation between day of sacrifice and %PF (Fig. 3D). Although the mean %PF for the moribund B10 mice in each experiment was 18.85% versus 38.30%, a simple statistical linear model shows no significant difference between experiments, once we account for the day of sacrifice. The model that accounts for the “day effect” has a P value of 0.0012. The %PF for transgenic mice was not different between the two experiments, 5.91 and 4.95 (P = 0.06). Survival differences between the two experiments also were not significant (P = 0.38).

Therefore, we can confidently combine the B10 data and test for differences in %PF between the three strains, A/J, B10, and B10.Ea. We used the linear model PctFib = \( \alpha + \beta_{\text{Strain}} + \epsilon \), with the constraint that \( \beta_{\text{A/J}} = 0 \). The overall P value for this model is \( 2.23 \times 10^{-10} \). The significance of the individual coefficients is 0.94 for the baseline \( \alpha \), 0.012 for the difference between baseline and transgenic mice, and \( 2.60 \times 10^{-11} \) for the difference between transgenic and B10 mice, indicating that the resistant A/J strain has nearly no fibrosis in response to bleomycin, the transgenic strain has a significantly higher response than does A/J, and the B10 strain has an extremely significant response over and above that of the transgenic strain. Thus, the transgenic strain shows considerable protection against pulmonary fibrosis, which further implicates H2-Ea as a strong candidate underlying Blmpf1.

Fig. 3. Bleomycin-induced pulmonary fibrosis in B10.Ea transgenic, control C57BL/10J, and A/J mice, the transgene donor strain. A, survival of C57BL/10J (n = 30), B10.Ea transgenic (n = 20), and A/J (n = 15) mice after 100 mg/kg bleomycin. B, %PF in individual C57BL/10J (n = 25), B10.Ea (n = 20), and A/J (n = 14) mice. C, %PF in end-of-experiment survivors; C, %PF in moribund mice sacrificed during the experiment. Bar, mean %PF for all mice in each strain. C, hematoxylin and eosin-stained left lung section from C57BL/10J and B10.Ea transgenic mice after treatment with 100 mg/kg bleomycin, with %PF for each section shown. D, %PF at the time of euthanasia for each mouse in the experiment. The cluster at day 56 is from mice that survived and were sacrificed at the end of the experiment. Solid line, the regression of %PF versus days for all mice; dashed line, regression of %PF versus days, only for those moribund mice that were sacrificed during the experiment. The actual slopes and P values for the significance of the slopes are given.
Discussion

We identified H2-Ea as a differentially expressed gene by using microarray, and we show that it is a candidate susceptibility gene for bleomycin-induced pulmonary fibrosis. It is known that pulmonary fibrosis is caused by abnormal immune responses to insults (4, 12), and it is well established that T cells play an important role in pulmonary fibrosis in mouse models (13–15). Thus, it is possible that H2-Ea and other class II MHC molecules are involved in lung injuries by activating CD4+ T cells.

Blmpf1 is a major locus that accounts for ~40% of the total genetic contribution to the trait and acts to increase susceptibility (2). The logarithm of odds score for this quantitative trait loci (QTL) is extremely high, 18, indicating the strong effect of this locus on the phenotype (2). QTL with strong contributions to diseases, such as Blmpf1, may actually contain multiple loci, e.g., the Idd3 locus for insulin-dependent diabetes (16) and Sle1 for systemic lupus erythematosus (17). Thus, in addition to H2-Ea, Blmpf1 may contain other genes contributing to susceptibility to bleomycin-induced pulmonary fibrosis. Using three MHC congenic strains B10.A-H2b4, B10.A-H2c5, and B10.A-H2o2, we narrowed Blmpf1 to a region of approximately 0.3 cM that includes H2-Ea as well as other potential candidates such as C4, C2, and other class II genes but not tumor necrosis factor.

We demonstrated that the MHC, specifically H2-Ea, is associated with susceptibility to bleomycin-induced pulmonary fibrosis in mice. However our data in the F2 and B10.Ea mice indicate that other genes, both non-MHC and MHC, may account for the persistence of fibrosis, albeit reduced, in the transgenic B10.Ea mice. A likely candidate is Blmpf2, a QTL on chromosome 11 that interacts with Blmpf1 and protects against fibrosis (2). Rossi et al. (5) showed that replacing the MHC H2o on the B10 background with H2b4, H2o, or H2a from resistant strains only partially reduced lung fibrosis, also suggesting the involvement of non-MHC genes. H2o, a dominant susceptibility loci, was the strongest genetic determinant of severe lupus nephritis in a backcross with MHC-congenic B6.H2o but showed no influence in a backcross with another MHC-congenic strain, BALB.H2o, which demonstrates that genetic background affects contributions from MHC susceptibility loci (18).

Our results may have relevance to human pulmonary fibrosis. Indeed, some MHC class II HLA are associated with human lung fibrosis (12, 19), although HLA-DRA, the human ortholog of mouse H2-Ea, has not been tested. Nevertheless, our results suggest a possible genetic basis for susceptibility to bleomycin-induced pulmonary fibrosis.

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