CLOSEOUT REPORT - RO1 OH007590

PI: Paul Brandt-Rauf, M.D., Sc.D., Dr.P.H.
Department of Environmental Health Sciences
Mailman School of Public Health
Columbia University
60 Haven Avenue
New York, NY 10032
TEL: 212-305-3464
FAX:212-305-4012
EMAIL: pwbl(u)columbia.edu

Institution: Columbia University in the City of New York
Health Sciences Division 630 West 168`h
Street New York, NY 10032

Project Title: P53 Biomarker and Intervention in Occupational Cancer

Date: November 20, 2007

Sponsor: NIOSH/CDC

Number: ROl0H007590

Dates: 06/01/02-05/31/06

Abstract

Research Methods for Occupational Cancer are needed to develop early markers of adverse health effects from workplace exposures and to devise ways for interrupting the pathways between workplace exposures and resultant cancers. The p53 tumor suppressor gene is a potential target for both of these approaches. Certain occupational exposures can produce mutations in p53 which cause the generation of an immune response with circulating p53 autoantibodies, even before the occurrence of clinically detectable cancers, so that these autoantibodies may serve as useful early markers of adverse effects. In addition, certain short protein sequences from p53 have been shown *in vitro* to cause mutant p53 to revert to normal function resulting in the death of cancer cells containing mutant p53, suggesting that this may be a useful approach for interrupting the pathway between workplace exposures that produce p53 mutations and resultant cancers. The purpose of this study was to examine both of these approaches for occupational cancers in two related projects.

In the first project, the presence of p53 autoantibodies was determined by ELISA in the banked serum samples from asbestosis cases collected between 1980 and 1987 and cancer mortality for the cohort was followed up to 2002. P53 autoantibodies were detected in 26. 5% of the individuals who subsequently developed cancer compared to 7.4% of the individuals who did not, a statistically significant difference (p=0.015), representing a positive predictive value of 0.76 and an average lead time to diagnosis of 3.5 years. In addition, in a repeat measures Cox proportionate hazards model, p53 autoantibody status was found to be highly statistically significantly predictive of subsequent. development of cancer (hazard ratio=5.5, 95%CI=2.8=10.9). Furthermore, p53 autoantibody status was found to be statistically significantly associated with p53 mutational status of the resultant tumors (Cohen's kappa=0.78, p=0.01). These results suggest that p53 autoantibodies are good predictors of cancer development in high-risk asbestosis cases and thus can be used as early markers of adverse health effects to identify those workers requiring more aggressive preventive interventions.

In the second project, we determined that a C-terminal p53 protein sequence (delivered as a peptide or as a mini-gene) was highly effective in producing apoptosis in premalignant and malignant cells with p53 mutations both *in vitro* in cell culture and in vivo in animal models via triggering of the FADD/caspase 8/caspase 3 pathway. In the animal studies, survival in the treatment groups was statistically significantly increased compared to the controls (p=0.01). Further structure-function studies of the protein sequence led to the development of an improved peptide with enhanced biological activity composed of a tetrameric palindrome of the C-terminal p53 sequence. Studies in *vitro* in cell culture and *in* vivo in animal models have confirmed the improved cytotoxic effect of this peptide in killing human mutant p53 cancer cells, including those that could develop from workplace exposures such as asbestos. These results suggest that this p53 peptide could be a novel and effective intervention for the chemotherapy or chemoprophylaxis of mutant p53 cancers produced by occupational carcinogens.

Highlights/Significant Findings

The first significant finding from this study is that p53 autoantibodies can predict the subsequent occurrence of cancer in high-risk workers with asbestosis. Therefore, these autoantibodies can serve as an early marker of cancer risk to identify those workers for more aggressive preventive interventions

The second significant finding from this study is that a short p53 protein sequence (delivered to cells as a peptide or as a mini-gene) can cause apoptosis of cells with a p53 mutation *in vitro* in cell culture and *in vivo* in animal models. Therefore, this p53 sequence can serve as a potential chemotherapeutic/chemoprophylactic intervention to treat or prevent cancers in workers who have experienced a p53 mutation from their workplace exposures (as identified by their p53 autoantibodies above).

Translation of Findings

These findings can be used to guide future investigative activities of high-risk cohorts for occupational cancer. For example, the early marker of carcinogenic effect could be used to identify individuals in the cohort who would benefit from early intervention with the specific preventive/curative therapy, and follow-up of the cohort could demonstrate a decrease in **cancer occurrence in these individuals.**

Outcomes/Relevance/Impact

The outcomes of this study would be classified as potential outcomes that could impact workplace risk if used. This study could lead to improvements in occupational health and safety because it identifies an early marker for occupational **cancer detection in high-risk workers and an intervention to prevent/treat occupational cancers in those high-risk workers.** As noted, the results of this study can thus be used to guide future studies that apply a combination of the early marker with the early intervention to document decreases in cancer incidence in high-risk cohorts.

Scientific Report

The background for this study was based on the objective of the National Occupational Research Agenda to develop new Research Methods for Occupational Cancer. These methods encompass the development of early markers of adverse health effects and approaches for interrupting the pathways between occupational exposure and resultant cancers. The p53 **tumor suppressor gene product was a logical target** for such methods development, since p53 is frequently mutated in occupational cancers which can lead to the development of an autoantibody response that can be detected prior to the clinical expression of disease (potentially serving as an early marker of carcinogenic effect), and since restoration of normal p53 function by a p53 peptide causes affected cells to undergo apoptosis (potentially serving as a way to interrupt the carcinogenic pathway and prevent disease). Thus, this study had two specific aims:

Aim 1: The first specific aim was to determine the relationship of p53 autoantibodies with the subsequent development of cancer in workers with asbestosis.

The methods used to achieve this aim relied on an ELISA assay to determine the p53 autoantibody status in 275 banked serum samples from 103 asbestosis cases collected between 1980 and 1987. We up-dated the cancer mortality status for these cases as of 2002. The results demonstrated p53 antibodies in 31 serum samples: in at least one serum sample in 13 of the 49 (26.5%) individuals who subsequently developed cancer (11 lung cancers, I mesothelioma, I other) compared to 4 of the 54 (7.4%) individuals who did not develop cancer, this difference was statistically significant (p=0.015), representing a negative predictive value of 0.58 and a positive predictive value of 0.76 with an average lead time to diagnosis (time from first positive sample to diagnosis) of 3.5 years (range=<1-12 years). In addition, in a repeat measures Cox proportionate hazards model, p53 autoantibody status was found to be highly statistically significantly predictive of subsequent development of cancer (hazard ratio=5.5, 95%CI=2.8-10.9). Furthermore, p53 autoantibody status was found to be statistically significantly associated with p53 mutational status of the resultant tumors (Cohen's kappa-0.78, p=0.01), confirming that the source of the cells that initially triggered the p53 autoantibody response were those that developed into the subsequent cancer. It was also possible to identify instances where both the tumor tissue and histologically normal tissue adjacent to the tumor contained areas of p53 alterations, further supporting the hypothesis that alteration in p53 occurred early in the carcinogenic process, prior to the development of overt malignancy, thus providing an explanation for the p53 autoantibody-positivity prior to clinical diagnosis of disease.

It is possible that the results for this cohort may even be stronger than described. For example, some of the cancer cases in this cohort developed well after 1988, the date of the last available serum samples, so it is quite possible that some of these cases developed p53 antibodies after 1988 but before their diagnosis of cancer but could not contribute to the risk relationship. Also, among the false-positive individuals (i.e., those antibody-positive who did not develop cancer), all 4 had only one positive sample which was always followed by a negative sample (as opposed to the true positives whose serum samples always remained positive once positive), and the antibody titers for these samples were just marginally positive by the *a priori* cut-off criteria (selected on the basis of prior studies as the level that best distinguished between cancer cases and matched non-cancer controls). Finally, the two individuals with positive samples who had not developed cancer as of the last follow-up may still develop cancer at some future date. Thus, the use of a higher cut-off for positivity, reliance on multiple positive samples to define a positive individual, access to additional serum samples after 1988, or longer follow-up time could improve the relationship between p53 antibodies and subsequent development of cancer even further. Nevertheless, the results as presented are the first to demonstrate a statistically significant relationship between p53 antibodies and subsequent cancer risk, and. they strongly suggest that p53 antibodies are good predictors of cancer development in high-risk asbestos workers and thus may be useful as early markers of cancer risk.

We have extended this analysis to include other biomarkers with p53 antibodies to estimate cancer risk to improve the sensitivity of testing which is relatively low for the p53 antibodies alone. For example, preliminary results combining all the biomarkers tested this far in this cohort with p53 antibodies not only retain high positive predictive value (0.76), negative predictive value (0.66) and specificity (0.85) but also increase the

sensitivity considerably (0.51). As additional biomarkers are developed, this could potentially be improved even further. To this end, we began to explore a proteomics approach to biomarker identification in this cohort. Using surface enhanced laser desorptionlionization - time of flight - mass spectrometry (SELDI-TOF-MS), we analyzed serum samples from 5 workers who developed cancer and 5 workers who did not develop cancer and tentatively identified 4 protein peaks that are highly discriminatory for cancer risk and are not consistent with any of the previously identified
biomarkers. In preliminary analyses, using the two best peaks as biomarkers in conjunction with the previously identified biomarkers increases the sensitivity for cancer detection to 0.90. Thus, combinations of other biomarkers with p53 antibodies could be even better at identifying high-risk asbestos workers requiring more aggressive preventive interventions in the future.

Aim 2: The second specific aim was to develop a mutant p53-specific intervention based on a p53 sequence (to be delivered as a peptide or as a plasmid mini-gene) to selectively kill cancer cells (as could occur in asbestosis cases, e.g., occupational lung cancers). The methods used to achieve this aim first required the establishment of a
suitable set of cell lines for study using a human, non-small cell lung cancer line (H1299) which is histologically similar to many of the lung cancers found in the asbestosis cohort but which is p53-null (this to be used as a negative control). This line was stably transfected with various plasmids: ~1) a plasmid that expresses a temperature-sensitive p53 mutant protein such that at 32.5 C the p53 acts like the wild-type protein but at 37°C acts like the mutant protein (the advantage of this is that we could compare the effects of the peptide therapy on cells that have no p53, wild-type p53 and mutant p53 but are otherwise identical); and (2) a plasmid that expresses a common mutant p53 protein that occurs in lung cancers from the asbestosis cohort (the advantage of this is that it will most closely mimic those cancers caused by asbestos). In addition, we obtained human malignant mesothelioma cell lines that contain normal levels of wild-type p53 or mutant p53 to use to mimic the mesotheliomas that can occur with asbestos exposure; however, these cells proved to be relatively resistant to peptide therapy due to a genetic defect in
the apoptosis pathway that is critical to the peptide's effect, so subsequent research focused on the lung cancer lines and other cancer lines that yielded a better response. In addition, the particular nature of the p53 sequence and method of delivery were investigated extensively and optimized to achieve the best cell killing. The p53 Cterminal peptide sequence was found to be quite effective in killing the mutant p53 H 1299 lung cancer cells and other cancer cells with mutant p53, as well as pre-malignant adenoma cell lines with mutant p53, but to have little to no effect on non-cancer cells with normal levels of wild-type p53. The mechanism of cell death was definitively
established to be apoptotic via the FADD/caspase 8/caspase 3 pathway.

In addition to fulfilling the original goals of demonstrating an effect on cells in culture, we have also been able to demonstrate *in vivo* results. We applied the C-terminal p53 peptide sequence in an existing p53-mutant rat cancer model based on delivery by intracerebral clysis. Tumors from control animals showed minimal apoptosis (3%) compared to 52% apoptosis in the peptide-treated animals, and the difference in the survival curves for the two groups was statistically significant (p=0.01). However, in further experiments on stably transfected H1299 lung cancer cell lines that expressed the C-terminal peptide from a plasmid-based mini-gene under the control of a tetracycline

promoter attempting to demonstrate the feasibility of this method of peptide delivery as originally proposed, it became evident that the intracellular half-life of the peptide was quite short which probably was limiting it full effectiveness. Subsequent structural studies attributed this short half-life to the lack of secondary structure of such a short peptide which predisposes it to rapid intracellular proteolytic degradation. It was noted that expression of a plasmid for the peptide linked to the larger GFP fluorescent marker protein (for locating the site of action of the peptide **in** cells) was much more stable. Therefore, we set about designing a plasmid for a longer peptide based on the original Cterminal p53 sequence but which would have greater secondary structure and hence more stability and greater cytotoxic effect. The C-terminal sequence was extended to amino acid residues 353 to 393 and repeated palindromically four times with glycine insertions between each repeat (i.e, 353-393-Gly-393-353-Gly-353-393-Gly-393-353). The glycines were inserted to provide flexibility between each repeat segment to allow for folding together of the segments, and the repeats were palindromically ordered so that folded together they would mimic the structure of the normal intact p53 tetramer with parallel alignment of its four C-terminal sequences. This palindromic tetramer construct (delivered either as a plasmid-based mini-gene or as a peptide with the ***Antennapedia*** leader sequence for membrane penetration) proved to be much more stable and much more effective at cell killing than the original peptide sequence. Because of the increased efficacy of the palindromic tetramer, this is now the treatment of choice, and a patent has been applied for based on delivery of this sequence either as a peptide or as a plasmid mini-gene. Preliminary animal studies using nude mice bearing the H1299 lung cancer cell implants indicate that delivery of the palindromic tetramer sequence as a plasmid mini-gene via an infusion of an adenovirus vector can achieve significant cell killing *in* vivo. Thus, we are well on the way to having a novel and effective intervention for the treatment and prevention of mutant p53 cancers that could arise from asbestos exposure or other causes.

In summary, the original specific aims were completely accomplished, and significant progress was made in extending the original aims.

Publications

1. Li Y, Rosal RV, Brandt-Rauf PW, Fine RL (2002). Correlation between hydrophobic properties and efficiency of carrier-mediated membrane transduction and apoptosis of a p53 C-terminal peptide. *Biochemical and Biophysical Research* ***Communications*** **298:439-449.**

2. Do TN, Rosal RV, Drew L, Raffo AJ, Michl J, Pincus MR, Friedman FK, Petrylak DP, Cassai N, Szmulewicz J, Sidhu G, Fine RL, Brandt-Rauf PW (2003). Preferential induction of necrosis in human cancer cells by a p53 peptide derived from the MDM2 binding site. *Oncogene* 22:1431-1444.

3. Brandt-Rauf PW, Rosal RV, Fine RL, Pincus MR (2004). Computational protein chemistry of p53 and p53 peptides. *Frontiers in Bioscience* 9:2778-2787.

4. Rosal R, Pincus MR, Brandt-Rauf PW, Fine RL, Michl J, Wang H (2004). NMR solution structure of a peptide from the mdm-2 binding domain of the p53 protein that is selectively cytotoxic to cancer cells. *Biochemistry* 43:1854-1861.

5. Li Y, Karjalainen A, Ksokinen H, Hemminki K, Vainio H, Shnaidman M, Ying Z, Pukkala E, Brandt-Rauf PW (2005). P53 autoantibodies predict subsequent development of *cancer. International Journal of Cancer* **114:157-160.**
6. Rosal R, Brandt-Rauf PW, Pincus MR, Wang H, Mao Y, Li Y, Fine RL (2005). The role of alpha-helical structure in p53 peptides as a determinant fro **their mechanism of cell death: necrosis versus apoptosis.** ***Advanced Drug Delivery Reviews*** **57:653-660.** 7. Li Y, Mao Y, Rosal RV, Dinnen RD, Williams AC, Brandt-Rauf PW, Fine RL (2005). Selective induction of apoptosis through the FADD/caspase-8 pathway by a p53 Cterminal peptide in human pre-malignant and malignant cells. *International Journal of Cancer* 115: 55-64.
8. Li Y, Mao Y, Brandt-Rauf PW, Williams AC, Fine RL (2005). Selective induction of apoptosis in mutant p53 pre-malignant and malignant cancer cells by PRIMA-1 through the c-Jun N-terminal kinas (JNK) pathway. *Molecular Cancer Therapeutics* 4:901-909. 9. Senatus PB, Li Y, Mandigo C, Nichols G, Moise G, Mao Y, Brown MD, Anderson RC, Parsa AT, Brandt-Rauf PW, Bruce JN, Fine RL (2006). Restoration of p53 function for selective Fas mediated apoptosis in human and rat glioma cells in vitro and in vivo by a p53 C-terminal peptide. *Molecular Cancer Therapeutics* 5:20-28. 10. Jin YJ, Wang J, Qiao C, Hei TK, Brandt-Rauf PW, Yin Y (2006). A novel mechanism for p53 to regulate its target gene ECK in signaling apoptosis. *Molecular Cancer Research* 4:769-778.
11. Michl J, Sharf B, Schmidt A, Huynh C, Hannan R, von Gizycki H, Friedman FK, Brandt-Rauf PW, Fine RL, Pincus MR (2006). PNC-28, a p53-derived peptide that is cytotoxic to cancer cells, blocks pancreatic cancer cell growth in vivo. *International Journal of* ***Cancer*** **119:1577-1585.**
12. Dinnen RD, Drew L, Petrylak DP, Mao Y, Cassai N, Szmulewicz J, Brandt-Rauf PW, **Fine RL (2007). Activation of targeted necrosis by a p53 peptide: a novel death pathway** which circumvents apoptotic resistance. *Journal of Biological Chemistry* 282:2667526686.

Inclusion of Gender and Minority Study Subiects, - Not applicable.

Inclusion of Children -Not Applicable. **Materials Available for Other**

Investigators, - Not applicable.