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14. ABSTRACT

We observed a severe dose and time dependent decrease in cell viability,via apoptosis, by JP-8 in a rat lung epithelial cell line (RLE-6TN) and in monoblastoid (U937) cells, characterized by caspase 3 activation, PARP cleavage and pro-apoptotic, cytochrome c released from mitochondria. Jurkat cells transfected with Bcl-xL, demonstrated increased survivability when exposed to JP-8. Exposure of human keratinocytes to JP-8 markedly downregulates the anti-apoptotic members of the Bcl-2 family, and upregulates the pro-apoptotic members. Treatment with JP-8 of human keratinocytes, grafted to form a human epidermis on nude mice, revealed cytoxicity and altered histology *in vivo* and in fibroblasts from PARP $^{(-/-)}$mice. CGH genomic hybridization revealed gains in regions of chromosomes 4, 5, and 14, as deletion of a region of chromosome 14 and in PARP$^{-/-}$ animals. DNA microarray analysis of PARP $^{-/-}$ mice indicated down-regulation sets of genes involved in the tight regulation of mitotic/cell progression, DNA replication, and chromosomal processing and assembly. Finally, p53 undergoes extensive poly(ADP-ribosyl)ation early in apoptosis. Degradation of poly(ADP-ribose) on p53 was observed and coincided with marked induction of the p53 responsive genes, *bax and fas.*

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FINAL REPORT

THE INVOLVEMENT OF POLY(ADP-RIBOSYL) ATION IN DEFENSE AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES

(AFOSR –F49620-98-1-0420)

JUNE 1, 2001

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FINAL REPORT

The Key Involvement of Poly(ADP-Ribosyl)ation in Defense Against Toxic Agents: Molecular Biology Studies

(AFOSR Grant F49620-98-1-0420)

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(3 YEARS: APRIL 1998 – March 31, 2001)

1. MECHANISMS OF PARP INTERACTIONS WITH DNA REPLICATION/REPAIR PROTEINS MULTI-ENZYME COMPLEX AND ENZYMES: RELEVANCE TO JP-8 TOXICITY AND APOPTOSIS:

One of the overall **Aims** of my laboratory, because of its importance on all projects and relevance to JP-8 toxicity, during the last period was to biochemically, molecularly, and mechanistically characterize the participation and interaction of PARP in a multi-enzyme replication/repair complex (MRC), which has been termed the "synthesome," with the proteins in this complex and how it may influence DNA replication *in vitro* and elucidate the significance of observed $p(ADP\text{-}R)^n$ of several of the enzymes found in this complex.

Purified replicative complexes from various cells that had been characterized previously for their ability to catalyze viral DNA replication *in vitro*, by our collaborations with R. Hickey and L. Malkas. We showed that PARP exclusively co-purifies through a series of centrifugation and chromatography steps with core proteins of an 18-21S multi-protein replication complex (MRC) from human HeLa cells, as well as with the corresponding mouse MRC from FM3A cells. The multienzyme complex was shown to contain PARP and DNA polymerases a and 6, DNA primase, DNA helicase, DNA ligase, and topo I and II, as well as accessory proteins such as PCNA, RF-C, and RP-A. The immunoblot analysis of MRCs from both cell types with monoclonal antibodies to poly(ADP-ribose) polymer (PAR) reveals the presence of approximately 15 p(ADP-R)n proteins, which were further confirmed to be DNA polymerase α, DNA topoisomerase I, and PCNA. Subsequently, more extensively identified in the next study performed during the last period. These recent results suggested to us that PARP may play a regulatory role within the replicative apparatus as a molecular nick sensor controlling the progression of the replication fork (or DNA repair replication) or to modulate enzymes or factors in the complex by directly associating with them or by catalyzing their p(ADP-R)n, or both.

Simbulan-Rosenthal, C. M. G., Rosenthal, D. S., Boulares, A. H., Hickey, R., Malkas, L., Coll, J., and **Smulson, M. E.** Regulation of the expression or recruitment of components of the DNA synthesome by poly(ADP-ribose). *Biochemistry* 37: 9363-9370 (1998).

Simbulan-Rosenthal, C. M. G., Rosenthal, D. S., Iyer, S., Boulares, H., and **Smulson, M. E.** Involvement of PARP and poly(ADP-ribosyl)ation in the early stages of apoptosis and DNA replication. *Mol. Cell. Biochem.* 193, 137-148 (1999).

In follow-up studies, we further explored the p(ADP-R)n of the synthesome proteins, particularly PCNA. We found that equal amounts of MRC-complexed and free forms of PCNA were detected by anti-PCNA immunoblot analysis; however, only the MRC-complexed form was p(ADP-R)^n—suggesting that this modification of PCNA may regulate its function within the MRC. We also found that NAD inhibited the activity of DNA pol α in the MRC, in a dose-dependent manner, whereas the PARP inhibitor, 3-A-B, reversed this inhibitory effect. The MRC from 3T3-L1 cells before and after 24 h of induction of a round of DNA replication was purified and also from cells depleted of PARP by antisense RNA expression (which do not undergo DNA replication or terminal differentiation).

The MRC, after 24 hr of differentiation induction in mock-transfected 3T3-L1 cells, expresses PARP, DNA pol α DNA primase and RPA. When antisense cells were examined, none of these proteins were expressed. Control cells exhibited a marked increase in the expression of E2F-1 as early as 1 h after induction of differentiation and DNA replication, consistent with the fact that the E2F-1 gene is an early response gene. PARP-depleted antisense cells are shown to contain negligible amounts of E2F-1 during the 24 h exposure to inducers of differentiation. Taken together, the results of this publication, indicate that either PARP protein, per se, or p(ADP-R)n, regulate the expression of DNA replication genes during early Sphase, indirectly by affecting the expression of the transcriptional factor, E2F-1. *This new information is relevant to exposure of cells or tissue to JP-8, since an error-prone DNA repair MRC may result. Additionally, error-prone repair caused by JP-8 could influence the anticipated CGH and SKY results (**Aim II**) orJP-8 induced gene expression changes (DNA array **Aim III*** of the new grant*)*. Alternatively, since we showed earlier that PARP depletion results in significant changes in chromatin structure (i.e. increased sensitivity to DNase I digestion; it is also possible that the effects of PARP depletion may be due to indirect alterations in chromatin structure.

Simbulan-Rosenthal, C.M., Rosenthal, D.S., Luo, R., **Smulson, M.E.** Poly(ADP-ribose) polymerase upregulates E2F-1 expression and promoter activity during entry into S-phase. Oncogene 18, 5015-5023 (1999).

In the most current work, we utilized the E2F-1 promoter sequence fused to luciferase cDNA in a plasmid, which was transiently transfected into wild-type PARP$^{(-/-)}$ and PARP$^{(+/+)}$, plus PARP cells. The cells were synchronized into S-phase (note: the PARP$^{(-/-)}$ cells lag in their entry into S-phase under these conditions. The PARP K/O cells, which had been retransfected with PARP cDNA (PARP $^{(-/-)}$ plus PARP) showed an 8-fold increase in their level of luciferase activity, indicating E2F promoter activity (see Fig. 16). Additionally, we performed DNA pol α expression studies using RT-PCR analysis, which demonstrated the presence of pol α RNA transcription in both control and K/O cells, retransfected with

PARP cDNA, 20 h after serum addition. These recent observations **establish an understanding** of whether PARP, or perhaps p(ADP-R)n proteins, activate E2F-1 promoter by cycling protein on an off of upstream sequences of E2F.

Smulson, M.E., Pang, D., Jung, M., Dimtchev, A., Chasovskikh, S., Spoonde, A., Simbulan-Rosenthal, C.M., Rosenthal, D., Yakovlev, A., and Dritschilo, A. Irreversible binding of poly(ADP-ribose) polymerase cleavage product to DNA ends revealed by atomic force microscopy: possible role in apoptosis. *Cancer Res.*, 58(16):3495-3498(1998).

Rosenthal, D. S., Ding, R., Simbulan-Rosenthal, C. M. G., Cherney, B., Vanek, P., and **Smulson, M. E.** Detection of DNA Breaks in Apoptotic Cells Utilizing the DNA-binding Domain of Poly(ADP-ribose) Polymerase with Fluorescence Microscopy. *Nucleic Acid Res.* 25: 1437-1441 (1997).

With the view of providing a more precise **mechanistic rationale** for a "cycling hypothesis" for the interaction of PARP with both proteins and DNA (which relates to the proposed studies in the new grant with JP-8 and apoptosis), we have recently explored the use of the technique Atomic Force Microscopy (AFM) in order to directly visualize PARP's binding to various forms of DNA; a theoretical advantage of AFM is that many of the morphological studies can be *performed in solution*, to allow, for example, incubation of PARP with its substrate, NAD, etc., and subsequently view its association or disassociation from DNA by this microscopic technique. AFM reveals that recombinant full length of PARP binds to plasmid (500 bp) restriction DNA fragments and links them into chain-like structures (Fig. 1B).

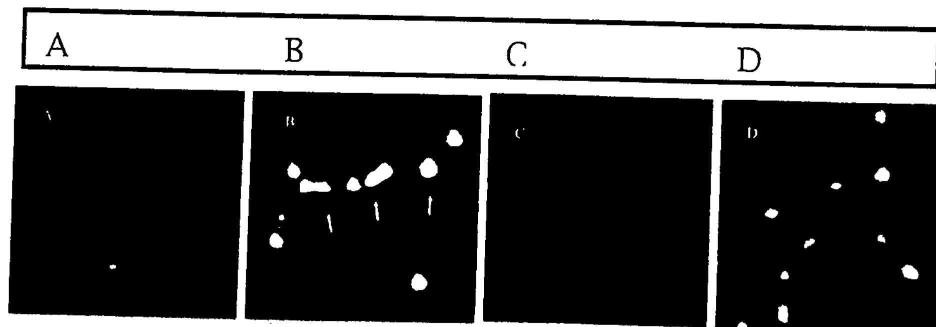

Figure 1

Automodification of PARP with NAD results in its dissociation from DNA fragments (Fig. 1C), identical to naked DNA control (Fig. 1A). In Fig. 1D recombinant PARP and DNA were incubated with NAD in the presence of 3AB, an inhibitor of PARP automodification. PARP still remains bound to the ends of the DNA structures. In this work, identical results were obtained on incubation of internucleosomal DNA fragments from apoptotic cells with the 2 peptides of cleavage of recombinant PARP by purified caspase-3. In this case, the 24-KDa/ DNA binding domain allows the binding of PARP

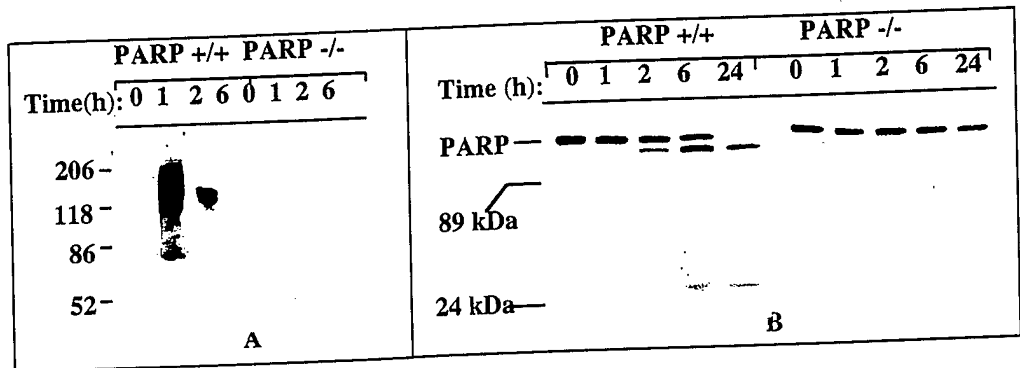

Figure 2

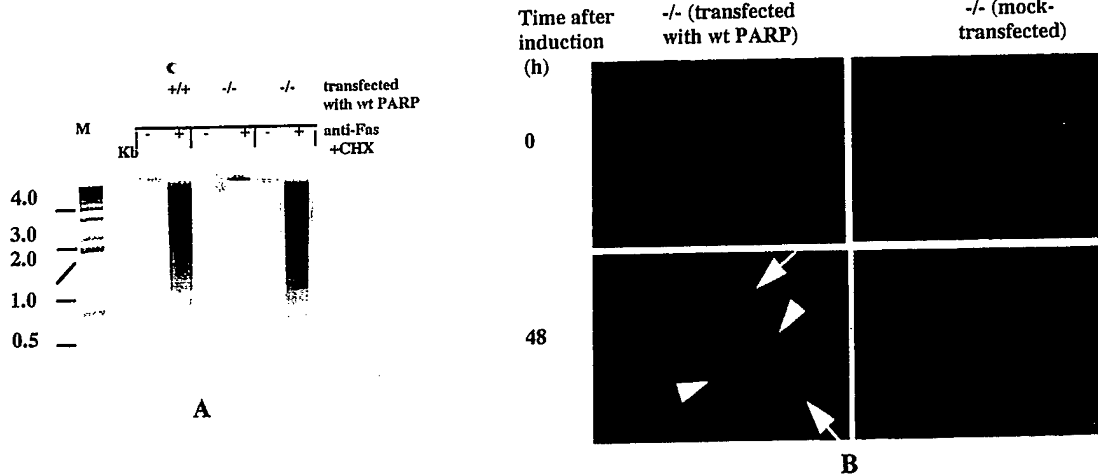

Figure 3

to apoptotic 200-400bp DNA fragments. In this case, as predicted, NAD and automodification had no influence on the continued binding of PARP to these DNA fragments.

In the second paper cited above, which was a basis for the past AFOSR grant of which this is the **Final Report**, a recombinant poly-his-tagged peptide, spanning the DNA binding domain of PARP was expressed, purified and used to detect DNA strand breaks in fixed cells. Fluorescence microscopy, with this probe using biotinylated PARP DNA binding domains was studied during the later stages of apoptosis in several cell systems when internucleosomal strand breaks became evident. This provides an alternative method to Tunnel Assays to visualize apoptotic DNA fragmentation.

2. MECHANISTIC ROLE FOR PARP/NAD IN APOPTOSIS: RELEVANCE TO JP-8 TOXICITY

Rosenthal, D. S., Ding, R., Simbulan-Rosenthal, C. M. G., Vaillancourt, J. P., Nicholson, D. W., and **Smulson, M. E.** Intact Cell Evidence for the Early Synthesis, and Subsequent Late Apopain-mediated Suppression of Poly(ADP-ribose) during apoptosis. *Exp.Cell Res.* 232: 313-321 (1997).

My involvement in this newly discovered role of PARP in cell death initially began as a collaboration between my laboratory and the Department of Medicinal Chemistry, Merck Research Laboratories of which Dr. Donald Nicholson is the head. Using the full-length human PARP cDNA clone, pcD12--isolated earlier with support, in part, from AFOSR—our lab developed a substrate ([^{35}S] PARP using *in vitro* transcription/translation) for an assay for the isolation of the human PARP cleavage enzyme.
Since most of the early research on PARP cleavage and apoptosis had at that time relied on *in vitro* assays, we decided to explore in more detail PARP's role in apoptosis, in the context of the whole cell by examining the various PARP participants in this aspect of programmed cell death by immunofluorescence of cells undergoing apoptosis. During each of the ten days of spontaneous apoptosis, we examined cells by immunofluorescence with antibodies to poly(ADP-ribose) polymer (PAR), PARP and 24kd PARP cleavage product of caspase-3; we also used the biotinylated PARP DBB assay described above to measure DNA strand breaks.

We observed an unexpected transient "burst" of poly(ADP-ribose) polymerase nuclear proteins and PARP expression only at day 3 prior to commitment to death, followed by caspase-3-mediated cleavage of PARP. *A transient early "burst" of PAR was also detected in JP-8 exposed lung cell cultures (see below).* It should also be noted in that in days 7 to 10, considerable breaks in DNA occurred. Since PARP activity had been cleaved by caspase-3 neither anti-PARP nor use of PAR antibodies stained cells, perhaps, illustrate a biological caspase-3 cleavage of PARP. This has been further pursued using PARP mutants incapable of caspase-3 cleavage. The identification of the limited and short activation of PAR in a transient and discrete time period during apoptosis, (day 3) was thus pursued in my lab for its generality in other cell systems in the next series of publications.

Simbulan-Rosenthal C.M., Rosenthal, D.S., Iyer, S., Boulares, A.H., **Smulson, M.E.** Transient poly(ADP-ribosyl)ation of nuclear proteins and the role of poly(ADP-ribose)polymerase in the early stages of apoptosis. *J. Biol.Chem.* 273(22:13703-13712(1998).

The occurrence or **elimination** of this early transient, p(ADP-R)n of nuclear proteins by depletion of PARP by antisense RNA expression or using other transfected PARP antisense cells and also cells derived from PARP K/O mice was thus examined with respect to various morphological and biochemical markers of apoptosis. Neither caspase-3-like activity, nor proteolytic processing of the inactive caspase-3 proenzyme (CPP32) to its active form; nor morphological changes or DNA ladders were apparent in PARP depleted 3T3-L1 antisense cells exposed to apoptosis inducers. A burst of PARP is observed in the control cells by 4 h, which was totally eliminated by pre-incubation of antisense induction. While immortalized $PARP^{(+/+)}$ fibroblasts showed the early burst of poly(ADP-ribosyl)ation and a rapid apoptotic response when exposed to anti-Fas and cycloheximide (Fig.2A, hour 1), $PARP^{(-/-)}$ fibroblasts did not exhibit either the early p(ADP-R)n (Fig. 2A) nor any biochemical, such as PARP cleavage (Fig. 2B, hours 0-6), or morphological changes (i.e. nuclear condensation) characteristic of apoptosis when similarly treated (Fig. 3B). However, stable transfection of $PARP^{(-/-)}$ fibroblasts with wild-type PARP cDNA renders the cells again sensitive to apoptosis, as shown by the re-emergence of internucleosomal DNA ladders (Fig. 3A, right) and nuclear condensation after 48 h. of anti-Fas and cycloheximide (Fig 3B).
We believe that this striking requirement for a transient, early activation of PARP is related to a restructuring of chromatin and large breaks in chromatin, which are known to occur early in apoptosis, and initial future experiments **obtained in the last Grant period** outlined in the current **Final Report** are focused on the follow-through of this hypothesis.

Boulares, A.H., Yakovlev, G., Ivanova, V., Stoica, B.A., Iyer, S., **Smulson, M.E.** Role of PARP cleavage in apoptosis: Caspase 3 resistant PARP mutant increases rates of apoptosis in transfected osteosarcoma cells. *J. Biol. Chem.*, 274: 22932-2294, (1999).

Based on support, in part, by AFOSR and in NCI Program Project (of which I am Co-PI), the role of PARP cleavage in apoptosis has been investigated in human osteosarcoma cells stably transfected with a vector encoding a caspase-3-resistant PARP mutant generated in our laboratory. Expression of this mutant PARP increases the rate of both spontaneous and staurosporine-induced apoptosis in osteosarcoma cells (as well as $PARP^{(-/-)}$ cells and $PARP^{(-/-)}$ cells stably transfected with PCR generated wild-type mutant, uncleavable PARP cDNA), at least in part by reducing the time interval required for the onset of caspase-3 activation and internucleosomal DNA fragmentation, as well as the generation of 50 kb DNA breaks, thought to be associated with early chromatin unfolding via changes in nuclease sensitivity (a topic to be performed during the current grant period). We also noted that NAD levels did drop during apoptosis in cells expressing this mutant, which cannot be cleaved, whereas wild-type PARP, which had been cleaved by caspase-3, did not indicate NAD lowering. It should be noted that the lowering of NAD/ATP by JP-8 has been implicated in the past with the pathology caused by this agent. *Thus, a useful new molecular PARP mutant has been generated for possible future studies in the current granting period*

3. PROGRESS ON P53 POLY(ADP-RIBOSYL)ATION DURING APOPTOSIS AND RELATED TOPICS: RELEVANT TO JP-8 INDUCED APOPTOSIS.

Simbulan-Rosenthal, C.M., Rosenthal, D. **Smulson, M.E**. Poly(ADP-ribosyl)ation of p53 during apoptosis in human osteosarcoma cells. *Cancer Res.*, 59:2190-2194 (1999).

In recently published work using the slow osteosarcoma cell apoptosis we quantitated both the levels of pS3, and more importantly, the levels as well as the time-frame of p(ADP-R)n of p53 during apoptosis of the osteosarcoma cells. The work also represents the first proof that p53, per se, is p(ADP-R)n in the context of the whole cell. The data show that p53 is significantly expressed from days 2-9 of spontaneous apoptosis; however, p53 becomes heavily p(ADP-R)n only during the early stages of apoptosis (i.e. seemingly during the "transient burst" of early apoptosis). Interestingly, as capase-3 activity increases (and PARP becomes inactivated), p53 is still present during days 5-9, yet the chains f PAR are removed from the protein. This was definitively proved by double immunoprecipitation experiments derived from the intact cell studies. We also made the interesting observation that the initiation of expression of the p53 inducible pro-apoptotic proteins, Bax—and also Fas—occurred at almost exactly the same time frame when p(ADP-R)n is cleaved from p53 during apoptosis in the system.

Simbulan-Rosenthal, C.M., Rosenthal, D.S., Ding, R., Bhatia, K., **Smulson, M.E**.. Prolongation of the p53 Response to DNA Strand Breaks in Cells Depleted of PARP by Antisense RNA Expression and Poly(ADP-ribosyl)ation of p53During Apoptosis. *Biochem. Biophys. Res. Com.*, 253: 864-868 (1998).

Cell cycle checkpoint controls may be very significant to the newly proposed analysis of **DNA array** of **Aim III** of the **new AFOSR grant, which began 4/1/01**. We showed that 3-AB prolonged the lifetime of p53 subsequent to carcinogenic and x-ray exposure of cells. To establish whether this was a nonspecific effect of 3-AB. We stably transfected a Burkitt cell line with an inducible PARP antisense construct. PARP content was depleted in the cells and subsequently the cells were exposed to 6.3 Gy 7-radiation. In non-induced antisense cells, the p53 concentration reached a max. 2 h after exposure to radiation and returned to control values, as expected by 4 h. In contrast, the p53 response in PARP-depleted cells peaked at four hours with the levels of p53 remaining elevated for up to 12 h after gamma-irradiation. These results indicated to us that PARP activity in part, determines the duration but not the magnitude of the p53 response to DNA damage.

Mandir, A.S., Przedborski, S., Jackson-Lewis, V., Wang, Z., Simbulan-Rosenthal, C.M., **Smulson, M. E.,** Hoffman, B.E., Guastella, D.B., Dawson, V.L., Dawson, T. M.. Poly (ADP-Ribose) Polymerase Activation Mediates MPTP-induced Parkinsonism. *Proc. Nat. Acad. Sci.,* 96:5774 - 5779 (1999).

Our recent interactions with the Division of Neuroscience at Johns Hopkins University (headed by Dr. Solomon Snyder), specifically with Dr. Ted Dawson, has yielded new approaches toward the study of PARP, which corresponds to a basic lowering of ATP and NAD in neuronal tissues. Since ATP and NAD

depletion and nitric oxide (NO) synthetase may be of importance to JP-8 toxicity (and, perhaps, JP-8 induced neurological complications) this study seemed quite relevant to the new work proposed in the new granting period. Ischernia and Parkinson's disease can both be independently induced in mouse models by procedures or chemicals, which activate nitric oxide and super oxide anion. In this collaboration it was observed that PARP K/O animals (but not PARP$^{(+/+)}$) are resistant to the neurotoxic and physiological symptoms of Parkinson's Disease, as caused experimentally by MPTP (presumably via NO). In PARP$^{(-/-)}$ mice, both NAD and ATP levels are maintained, allowing for protection of neurological tissues, as well as an inability to progress into apoptosis. There have been three significant findings in studies performed in our laboratory with tissues derived from these animals and the p(ADP-R)n of a number of neuro-nuclear proteins subsequent to MPTP and the development of Parkinson's Disease. In PARP K/O animals—given MPTP—obviously no p(ADPR)n occurred. In NO synthetase K/O mice, no p(ADP-R) occurs, after administration of the neuro-toxin, MPTP, suggesting that NO is required, in some way, presumably through DNA damage. Further, our Westerns with anti-PAR (and anti-p53) in PARP$^{(+/+)}$ animals show that p53 is a major PAR acceptor molecule, after MPTP in neuronal tissues. We have not observed such a large level of p53 modification in any biological system yet examined.

Stoppler, H., Stoppler, M.C., Johnson, E., Simbulan-Rosenthal, C.M., Iyer, S., **Smulson, M.E.**, Rosenthal, D. S., and Schlegel, R. The human papillomavirus 16 (HPV-16) E7 protein sensitizes primary human keratinocytes to apoptosis. *Oncogene*, 17:10 12071214 (1998).

Cuvillier, O., Rosenthal, D.S., **Smulson, M.E.**, and Spiegel, S. Sphingosine l-phosphate Inhibits Activation of Caspase-3 that Cleave Poly(ADP-ribose). Polymerase and Lamins during Fas-and Ceramide-mediated Apoptosis in Jurkat T Lymphocytes. *J. Biol. Chem.* 273: 2910-2916 (1998).

In these two collaborative studies, performed with colleagues at Georgetown during the last period, we have made contributions in two novel apoptotic models. In the first paper our lab used the [^{35}S] PARP cleavage assay to help show that HPV-16 factor E6 does not suppress apoptosis in normal keratinocytes. In contrast, E7 increases both spontaneous and induced apoptosis as well as the cellular levels of p53 and p21.

The second paper reflects an ongoing effort between my laboratory and the neighboring laboratory of Dr. Sarah Spiegel. The study involves the role of SPP, a protein kinase activator, on C2 ceramide induced activation on the proteolysis of PARP and lamins, as well as the conversion of CPP 32 to caspase-3.

4. THE PARP K/O PHENOTYPE CAUSES SPECIFIC GENOMIC GAINS AND LOSSES IN MICE AND DERIVED CELLS: RATIONALE FOR AIM II OF CURRENT NEW AFOSR PROGRAM

Simbulan-Rosenthal, C.M., Haddad, B., Rosenthal, D.S., Weaver, Z., Coleman, A., Luo, R., Young, H., Wang, Z.Q., Ried, T., & **Smulson., M.E**. Chromosomal Aberrations in PARP$^{(-/-)}$ Mice:Genome Stabilization in Immortalized Cells by Reintroduction of PARP cDNA. *Proc. Nat. Acad Sci.* 96:13191-13196 (1999).

[This recent paper has directly stimulated the new approaches outlined in ***Aim II*** *of the new grant (3/1/01). By collaborations with Drs. Haddad and Ried, presently or formerly at the National Human Genome Institute, we have established CGH as an ongoing technique in our laboratory. This technique will allow us to test whether, over time, individual* <u>*chromosomal abnormalities*</u> *are induced in primary fibroblasts isolated from humans, exposed over long terms to JP-8 fuel vapor, or cells in culture exposed to non-apoptotic levels of J-P8. CGH will examine losses and gains of regions of chromosomes, while SKY-Spectral Karyotyping, (i.e. chromosome painting) will allow us to examine chromosomal translocations.]*

Depletion of poly(ADP-ribose) polymerase (PARP) increases the frequency of recombination, gene amplification, sister chromatid exchanges, and micronuclei formation in cells exposed to genotoxic agents, implicating PARP in the maintenance of genomic stability. Flow cytometric analysis revealed an unstable tetraploid population in immortalized fibroblasts derived from PARP$^{(-/-)}$ mice, using comparative genomic hybridization (CGH) we detected partial chromosomal gains in three specific chromosomes, 4C5-ter, SF-ter, and 14Al-C1 in PARP$^{(-/-)}$ mice and also using total DNA isolated from immortalized PARP$^{(-/-)}$ fibroblast cultures. Neither the chromosomal gains nor the tetraploid population were apparent in PARP$^{(-/-)}$ cells stably transfected with PARP cDNA (PARP$^{(-/-)}$, +PARP), indicating negative selection of cells with these genetic aberrations after reintroduction of PARP cDNA. While the tumor suppressor p53 was not detectable in PARP$^{(-/-)}$ cells, p53 expression was partially restored in PARP$^{(-/-)}$, +PARP) cells. We also detected specific loss of 14D3-ter that encompasses the tumor suppressor gene Rb-1 in PARP$^{(-/-)}$ mice. This loss (among others at this loci) was associated with a reduction in Rb expression; increased expression of the oncogene Jun was correlated with a gain in 4C5-ter that harbors this oncogene. These results further implicate PARP in the maintenance of genomic stability, and suggest that altered expression of p53, Rb, and Jun may further contribute to genomic instability associated with PARP deficiency.

5. RECENT STUDIES ON THE MOLECULAR MECHANISMS FOR JP-8 INDUCED CELL KILLING IN RAT LUNG EPITHELIAL CELLS, MONOBLASTOID CELLS, JURKAT-T CELLS AND IN HUMAN SKIN LAYERS AND HUMAN KERATINOCYTES IN CULTURE.

Stoica, B.A., Boulares, A.H., Rosenthal D.S., Iyer, S., Hamilton, I.D.G., **Smulson, M.E.**. Mechanisms of JP-8 jet fuel toxicity: I. Induction of apoptosis in rat lung epithelial cells. *Tox. Appl. Pharmacol.*, 171(2):94-106. (2001)

Inhalation is one of the primary routes of JP-8 exposure, therefore we decided to establish an *in vitro* model that will allow the dissection of the mechanisms underlying the JP-8 jet fuel toxicity in a cell line representative for the respiratory system. Therefore we investigated the effects of JP-8 on a lung epithelial cell line (RLE-6TN). RLE-6TN is a cell line that originated from rat alveolar epithelial cells and preserves many of the phenotypic characteristics of the original alveolar cells. It was observed that JP-8 induces a

dose-dependent decrease in cell viability in the RLE-6TN cells after 24h of exposure at the indicated dilutions of JP-8. While a $1x10^{-4}$ dilution of JP-8 is able to kill virtually all the cells, lesser but reproducible effects were noted as the JP-8 dilution was increased up to $1.25x10^{-5}$ (Fig.4A). In order to evaluate the death rate we measured cell viability during a time-course exposure to a $1x10^{-4}$ dilution of JP-8 jet fuel. The results indicate a rapid decrease in cell viability with a little more that 50% of the cells being dead after six hours of exposure to JP-8 (Fig.4B).

In order to gain further insight in the type of cell death induced in RLE-6TN cells upon exposure to 8 we evaluated cellular morphology by phase contrast microscopy during a time course of treatment with a $1x10^{-4}$ dilution or JP-8 jet fuel. When exposed to JP-8 the earliest cellular change noted was that the normally adherent RLE-6TN cells became rounded between 2 h and 4 h. This was followed by membrane blebbing, fragmentation and detachment from the substrate after 6 h. After 8 h of treatment most of the cells were fragmented and floating (data not shown).

An exposure to JP-8 as short as 2 h induces a detectable increase in caspase-3-like activity with a peak being reached after about 4 h of exposure. A gradual decrease in activity occurs thereafter (Fig. 5A). This pattern of caspase-3 activation is typical of apoptosis. Caspase-3 activation occurs when the inactive caspase proenzyme of 32 kDa (p32) is specifically cleaved into a large subunit of 20 kDa (p20) and a small subunit 12 kDa (pl2). Cell extracts originating from RLE-6TN cells treated with JP-8 for various times were used for an immunoblot analysis with an antibody specific for the pl7/20 fragment (Fig. 5B). The presence of the p20 subunit was detected as early as 4 h after treatment. The intensity of the p20 band increased further between 6 h and 8 h. In parallel, there is a decreased intensity of the band corresponding to the p32 caspase-3 precursor. In RLE6TN cells treated with JP-8 the p89 cleavage fragment of PARP is detectable, by Western blot, at the 4 h time point and its abundance increases at the 6 h time point (Fig. 5C). In parallel, there is a relative decrease in intensity of the pl16 (i.e. PARP). **Mitochondrial injury is an important inducer of apoptosis**. Upon injury, mitochondria may release cytochrome c from the intermembrane space into the cytosol where it can activate caspase-9, via Apaf I and ATP/dATP. In order to investigate the possibility that **JP-8 treatment causes apoptosis through a mitochondrial damage pathway**, we examined the release of cytochrome c into the cytosol following exposure of RLE-6TN cells to JP-8. When cytosolic extracts of cells exposed to JP-8 were probed with an anti-cytochrome c antibody, a large increase in cytochrome c levels in the cytosol fraction was observed as early as 2 h after JP-8 treatment (Fig. 5D). The levels of cytosolic cytochrome c continued to be elevated at later time points. This finding indicates that mitochondrial damage is one of the earliest manifestations of JP8 toxicity and occurs before caspase activation. Therefore it is possible that the apoptotic program is initiated due to this initial **mitochondrial damage**.

The human monoblastoid cell line (U-937) responds to JP-8 exposure with caspase-3 activation, cleavage of caspase substrates like PARP, DNA-PK, and lamin B1 and degradation of genomic DNA with the production of HMW fragments.

After 24 h of treatment with various dilutions of JP-8, cellular viability is decreased in a dose-dependent manner. A dilution of $1x10^{-4}$ killed all the cells, while a lesser effect was noted with increasing the dilutions (Fig. 6A). As in the case of RLE-6TN cells, it was important to establish if the cellular death

JP-8 induces a dose dependent and time dependent decrease in viability in RLE –6TN cells.

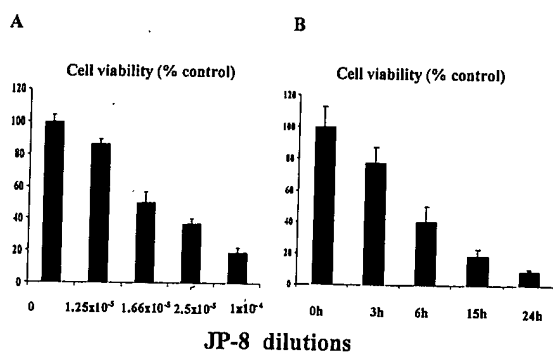

Figure 4

JP-8 induces Caspase 3 activation and Cytochrome C release in RLE-6TN cells.

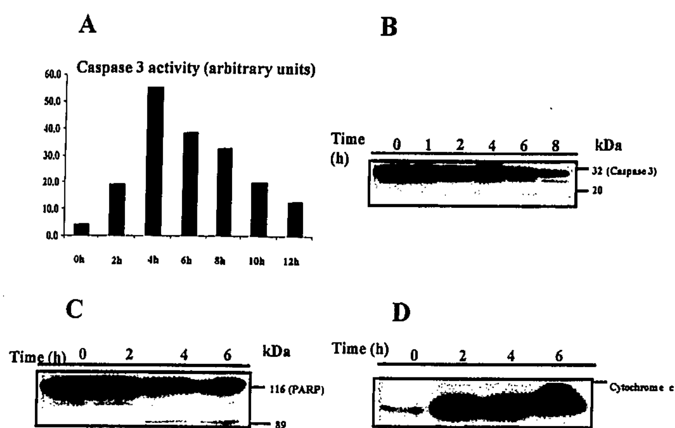

Figure 5

followed an apoptotic pattern. Caspase-3-like activity was examined in cell extracts obtained from U-937 treated with a lx10^{-4} dilution of JP-8 for various times using the fluorometric assay. 10^{-4} dilution of JP-8 for various times (Fig. 6B) show that a caspase-3-like activity is detectable as early as 1 h with an activity peak at the 2 h and a decrease thereafter. p32 (pro-caspase) cleavage is illustrated in Fig. 7A. The active p20 fragment of caspase-3 is visible as early as 1 h after JP-8 treatment and even more clearly after 2 h of treatment. In order to evaluate the integrity of the apoptotic pathways downstream of caspase-3 activation, we investigated by Western blot the cleavage of several proteins that are well-known caspase substrates. In Fig. 7B the appearance of the 89kDa fragment of PARP is evident as early as after 1 h of treatment. Lamin B1, an essential component of the nuclear lamina, is another substrate for caspase-6 during apoptosis. As presented in Fig. 7C, the cleavage of lamin B1 from its normal size of 64 kDa into the 46 kDa fragment occurs as early as 2 h after the start of JP-8 treatment. DNA-PK, which is an enzyme involved in DNA repair and recombination, is another substrate of caspase-3. As indicated in Fig. 7D, the cleavage of the catalytic subunit of DNA-PK from the full length of 460 kDa into 240 kDa and 160 kDa fragments is observable as early as 1 h after JP-8 treatment.

JP-8 induces a dose dependent decrease in viability and Caspase 3 activation in U-937 cells.

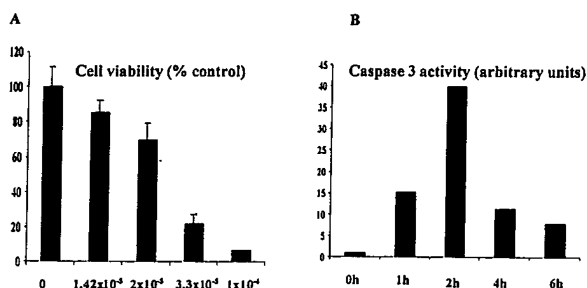

Figure 6

JP-8 induces the biochemical features of apoptosis in U-937 cells.

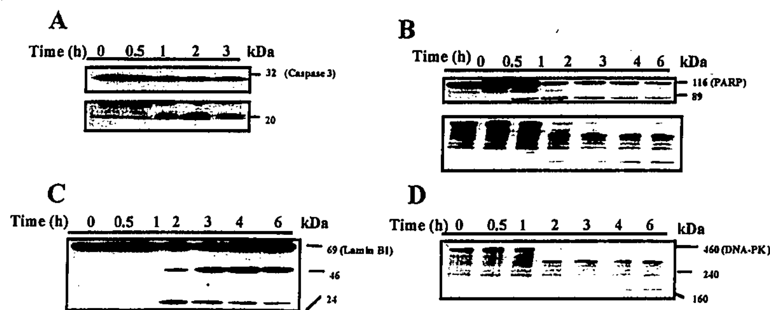

Figure 7

Caspase-3 activation and PARP cleavage are also induced in the Jurkat T cell leukemia cell line when treated with JP-8. Jurkat cells stably transfected with a plasmid expressing the anti-apoptotic protein Bcl-xL or pretreated with the D-Boc-Fmk caspase inhibitor demonstrate increased survivability when exposed to JP-8. These observations demonstrate that JP-8 is causing apoptotic cell death in several different cell lines, potentially by causing mitochondrial injury and that the inhibitors of apoptosis diminish JP-8 toxicity.

Rosenthal, D.S., Simbulan-Rosenthal, C.M.G., Liu, W.F., Stoica, B.A., and **Smulson, M.E.** Mechanisms of JP-8 jet fuel cell toxicity: II. Induction of necrosis in skin fibroblasts and keratinocytes and modulation of levels of Bcl-2 family members. *Tox. Appl. Pharmacol. 1;171(2):107-16. (2001)*

As shown in the manuscript above, JP-8 induces apoptosis in rat lung epithelial cells, primary mouse T lymphocytes, Jurkat T lymphoma cells, and U937 monocytic cells (25). Here, we have observed a different mechanism of cytotoxicity, by JP-8, on human keratinocytes grown in culture, as well as when

grafted onto nude mice. As shown in Fig. 8, at lower levels of JP-8 (0.08 μg/ml; 1×10^{-4} dilution), sufficient to induce apoptosis in other cell types, including alveolar (RLE) epithelial cells (25), no apoptosis was observed in either immortalized (Imm HEK) **or primary** (1^0 HEK.) human keratinocytes. At higher levels (>0.2 μg/ml; 2.5×10^{-4} dilution), JP-8 is cytotoxic to both primary and immortalized human keratinocytes, as evidenced by the metabolism of calcein, as shown by the data in Fig. 9 by morphological changes such as cell rounding and cell detachment.

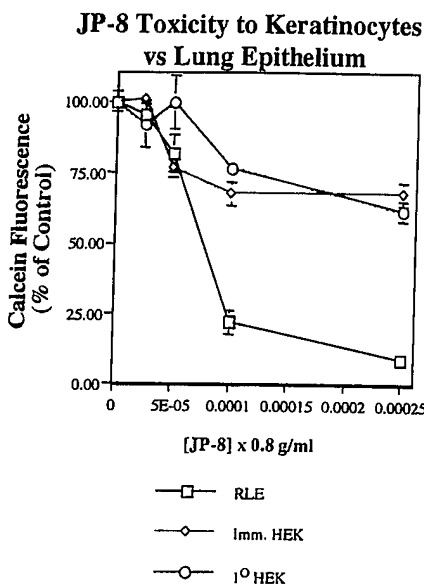

Figure 8

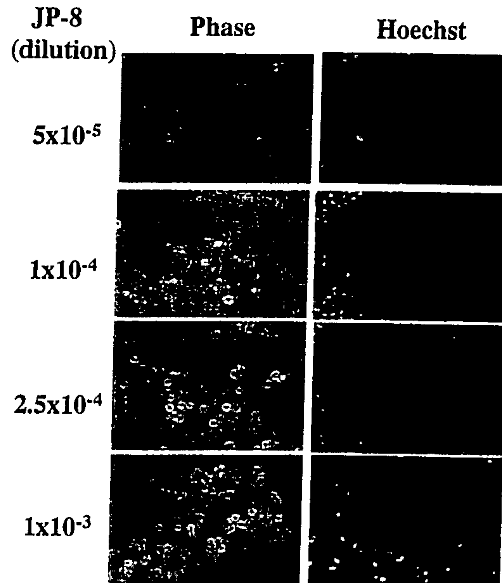

Figure 9

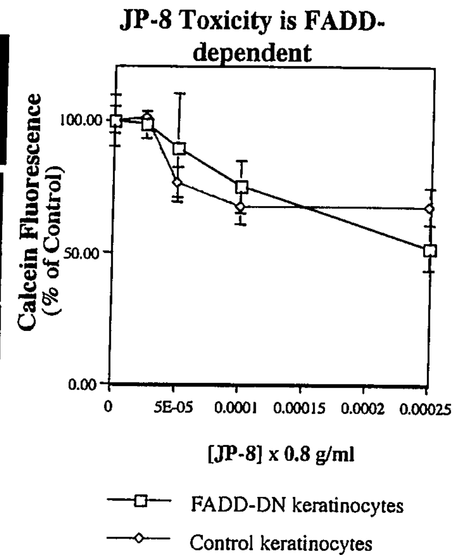

Figure 10

FACS analysis indicated that the majority of toxicity resulted from necrosis rather than apoptosis at these levels. In addition, there was no evidence of activation of caspases-3, -7, or -8 either by enzyme activity or immunoblot analysis.

FADD has also been shown to play a role in death receptor-mediated apoptosis by activating a pathway leading to the activation of caspases. However, even in the absence of caspase activity and DNA fragmentation, FADD has still been shown to initiate cell death by necrosis (26). To determine if the FADD pathway modulated the response of keratinocytes to JP-8, we stably transfected lines of Nco keratinocytes with a plasmid expressing the dominant-negative form of FADD (FADD-DN). These stable lines were then treated with JP-8 at the same concentrations described for Nco above. Treatment of cells, followed by cell viability analysis, revealed that the dose-response was independent of the expression of FADD-DN (Fig. 10).

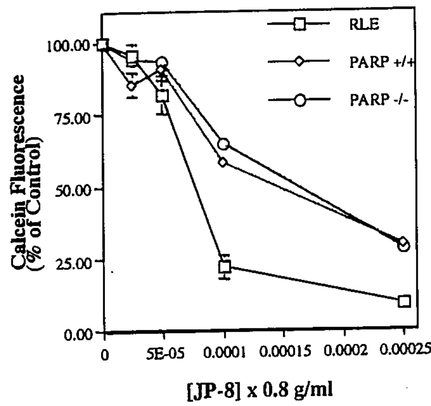

Figure 11

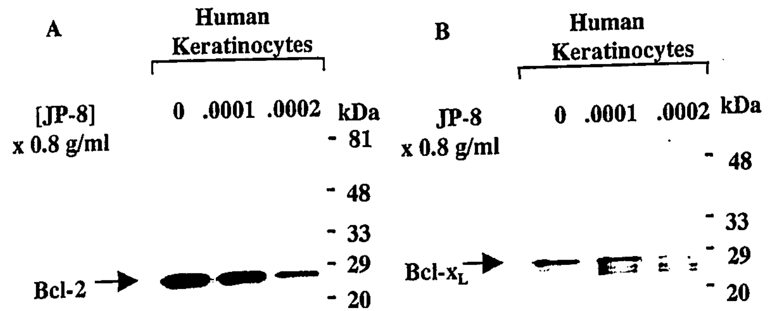

Figure 12

PARP has also been implicated in necrotic cell death. To determine the potential role of PARP in JP-8-mediated toxicity, **we utilized primary skin fibroblasts derived from PARP**$^{(-/-)}$, as well as PARP$^{(+/+)}$ mice. We found that the presence of PARP had no effect on the sensitivity of cells to JP-8 toxicity or chromatin condensation (Fig. 11). As in the case of immortalized keratinocytes, no concentration of JP-8 induced caspase-3 activity or processing from the 32-kDa proenzyme. In addition, immunoblot analysis revealed no JP-8 induced processing of either caspase-7 or caspase-8.

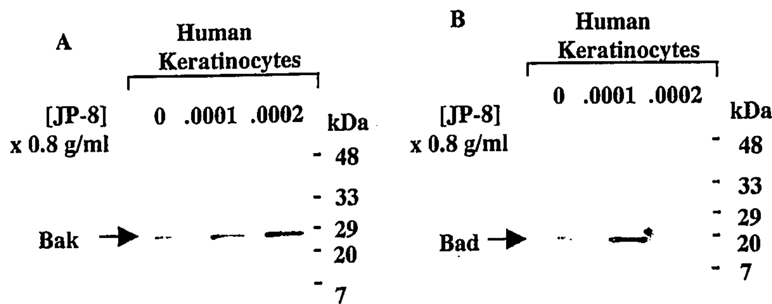

Figure 13

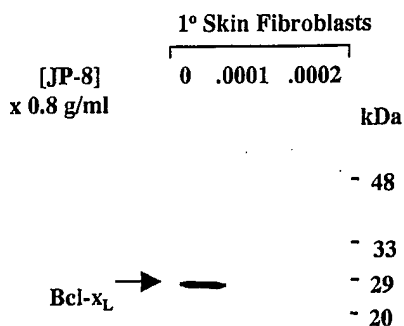

Figure 14

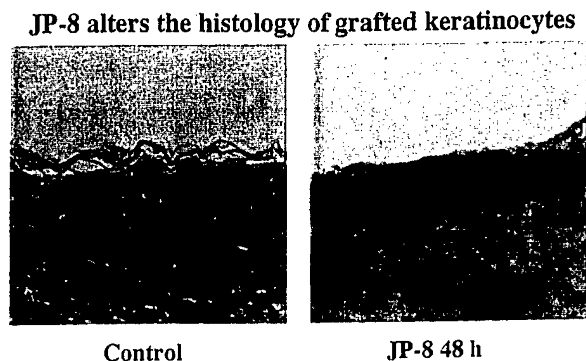

Figure 15

To determine if Bc1-2 proteins played a role in keratinocyte death induced by JP-8, immortalized keratinocytes were exposed to different concentrations of JP-8, fixed after 24 h, and then subjected to immunoblot analysis, using Bcl-reactive antibody as a tag. **Following JP-8 exposure, the level of Bc1-2 decreased significantly** (Fig. 12A). We were curious to determine if JP-8 altered the expression of any other Bc1-2 related proteins. Immunoblot analysis with specific antisera revealed that Bc1-xL was also reduced in the presence of JP-8 (Fig. 12B). We also examined the expression of Bak, a pro-apoptotic member of the Bcl-2 family that causes apoptosis via depolarization of the mitochondria. In extracts derived from untreated cells, Bak was not expressed. Interestingly, following 24 h exposure to JP-8, the level of Bak was significantly increased (Fig. 13B). We next examined the levels of Bad, expressed in Nco following JP-8 treatment. Immunoblot analysis revealed that the levels of Bad were also elevated following JP-8 treatment (Fig. 13A). Similarly, in primary skin fibroblasts, a decrease in the levels of Bcl-xL was observed following exposure to JP-8 (Fig. 14).

Immunoblot analysis thus revealed that exposure of keratinocytes to the toxic higher levels of JP-8 markedly down-regulates the expression of the pro-survival members of the Bc1-2 family, Bc1-2 and Bc1-xL, and upregulates the expression of anti-survival members of this family, including Bad and Bak. Bc1-2 and Bcl-xL have been shown to preserve mitochondrial integrity and suppress cell death. In contrast, Bak and Bad, both promote cell death by alteration of the mitochondrial membrane potential, in part by heterodimerization with and inactivation of Bcl-2 and Bcl-xL, and either inducing necrosis, or activating a downstream caspase program. High intrinsic levels of Bcl-2 and Bcl-xL may prevent apoptotic death of keratinocytes at lower levels of JP-8, while perturbation of the balance between pro-and anti-apoptotic Bc1-2 family members at higher levels may ultimately play a role in necrotic cell death in human keratinocytes.

Utilizing the grafting system as previously described, we grafted Nco cells on nude mice to form a histologically and immunocytochemically normal human epidermis. Fifty μ of JP-8 was then applied topically to the graft site. Fig. 15 shows the result of an experiment in which control tissue, or tissue

treated with topical JP-8, was isolated and subjected to histological analysis. In contrast to the normal morphology of control grafts, JP-8 treated skin showed gross histological abnormalities characterized by severe necrosis of all epidermal layers.

CONCLUSION

Update of Progress accomplished from August, 2000 (when current active AFOSR grant was submitted) until March 31, 2001 (end of earlier granting period)

Two major lines of research were accomplished during this period.

1. We completed experiments requested by reviewers and commenced through the usual publication procedures in writing, re-writing and proof reading the two papers we published on JP-8, and apoptosis (outlined in detail above) in Toxicology and Applied Pharmacology.

2. Second, we completed and published:

   Simbulan-Rosenthal, C.M., Ly D.H., Rosenthal D.S., Konopka, G., Luo R., Wang, Z.Q., Schultz, P. G., **Smulson, M.E.** Misregulation of Gene Expression in Primary Fibroblasts Lacking Poly(ADP-ribose) Polymerase. *Proc. Nat. Acad. Sci. USA* 97:11274-11279 (2000). This work on DNA array analysis of expression in PARP $^{+/+}$ and PARP $^{-/-}$ cells, formed the basis for the experiment we are now just initiating in combination with JP-8. Accordingly, Aim 2 of the new AFSOR grant, started April 1, will be summarized below.

Preparation of mRNA and Conversion to cDNA of Samples from Various Cells Utilized in this Study, as well as Fibroblasts from Human Volunteers.

The data, mutually obtained by my group and Scripps, will be outlined on expression changes due to the PARP K/O genotype to clarify the types of experiment and appropriate methods we are now initiating to assess expression changes due to JP-8 exposure, first in cells and subsequently with fibroblasts from workers exposed to this agent.

Representative Data obtained from August, 2000 – approximately March 2001 and Methods for Interpretations of Array Analysis Anticipated for JP-8 Exposure. As noted, DNA micro-array data methods used and data interpretation from an ongoing PARP K/O array analysis will be briefly discussed again as an indicator of the types of analysis (but not necessarily the same gene expression changes) anticipated for the future JP-8 experiments. In summary, during this period, we found that PARP deficiency significantly alters the expression of 63 genes (Table 1) and selectively down-regulates a set of genes involved in the tight regulation of mitotic/cell cycle progression (i.e. cyclins A and B, and $p55^{cdc}$), DNA replication (i.e. DNA primase, PCNA), and chromosomal processing and assembly (i.e. HMG-2).

genes involved in the tight regulation of mitotic/cell cycle progression (i.e. cyclins A and B, and $p55^{cdc}$), DNA replication (i.e. DNA primase, PCNA), and chromosomal processing and assembly (i.e. HMG-2). This suggests that mitotic misregulation in PARP deficient cells can cause increasing errors in the mitotic machinery of dividing cells, potentially leading to genomic alterations, such as formation of unstable tetrapoloid and aneuploid cells, predisposed to chromosome segregation abnormalities, as well as the partial chromosomal gains and losses in the PARP $^{(-/-)}$ cells and mice. It is also presumably this mitotic errors that may further lead to altered expression of subsets of genes that may likely contribute to genomic instability, cancer, and aging.

In these preliminary exploratory experiments, interesting parallels were noted between down-regulated genes in PARP $^{(-/-)}$ cells already noted in our CGH paper and alterations of gene expression, such as reduced expression of the same cell cycle regulatory genes involved in spindle assembly and chromosome segregation (cyclins A and B, $p55^{cdc}$/cdc 20, and HMG-2). Genes coding for extracellular matrix or cytoskeletal components implicated in cancer initiation and progression and in normal or premature aging were also up-regulated in PARP-null cells. These results suggest important mechanisms that may relate to selection processes that occur during cancer progression and aging, and that decreased PARP expression may be an early factor in the pathogenesis of cancer and age-related diseases. JP-8 presumably <u>may respond in much different</u> expression patterns.

Gene expression patters for $PARP^{-/-}$ cells was compared with wild-type cells (baseline), and only those changes that were reproducible in independent replicates were considered further, <u>*as will be the criteria for JP-8 data.*</u> Genes that were up- or down regulated with a differential expression > 2 are listed in Tables 1 and 2. Of the 10,000 genes monitored, 93 genes (~1.5%) showed consistent expression levels changes more than twofold. Changes in expression of a number of the down- as well as up-regulated genes were also verified at the protein level immunoblot analysis and confirmed (Fig. 28). Interestingly, the observed differences for the tested genes by immunoblot analysis appeared to be significantly higher than values of different expression determined by cDNA microarray analysis, suggesting that cDNA array hybridization may underestimate the magnitude of changes in gene expression. *I will thus stress this "double-back up" analysis in the **JP-8 study** also. It is an important control not always performed.* Twenty-three genes and 5 ESTs (35%) were identified by cDNA array as down-regulated and 50 genes and 13 ESTs (65%) were up-regulated in PARP-deficient cells, with a differential expression of ~2 –8 fold down-regulation or ~ 2 – 44 fold up-regulation (Table 1, 2).

Of the 93 genes that had altered expression as a result of PARP deficiency, about half can be grouped into 1) genes whose products are involved in the critical regulation of cell cycle progression (14%), and 2) genes involved in maintenance and remodeling of cytoskeletal and extracellular matrix (ECM) (26%) (Tables 1 and 2). A subset of genes involved in mitosis and regulation of cell cycle progression are down-regulated between 2- to 3- fold and include cyclins A, B1, and B2, which are typically up-regulated at the G_2-M phase and tightly regulate cell cycle progression. Consistent with the results of cDNA microarray

hybridization, immunoblot analysis of cell extracts with antibodies to cyclin A and B1 revealed a dramatic reduction in constitutive expression of cyclins A and B1 in PARP $^{-/-}$ cells relative to that in wild-type cells (Fig. 28A).

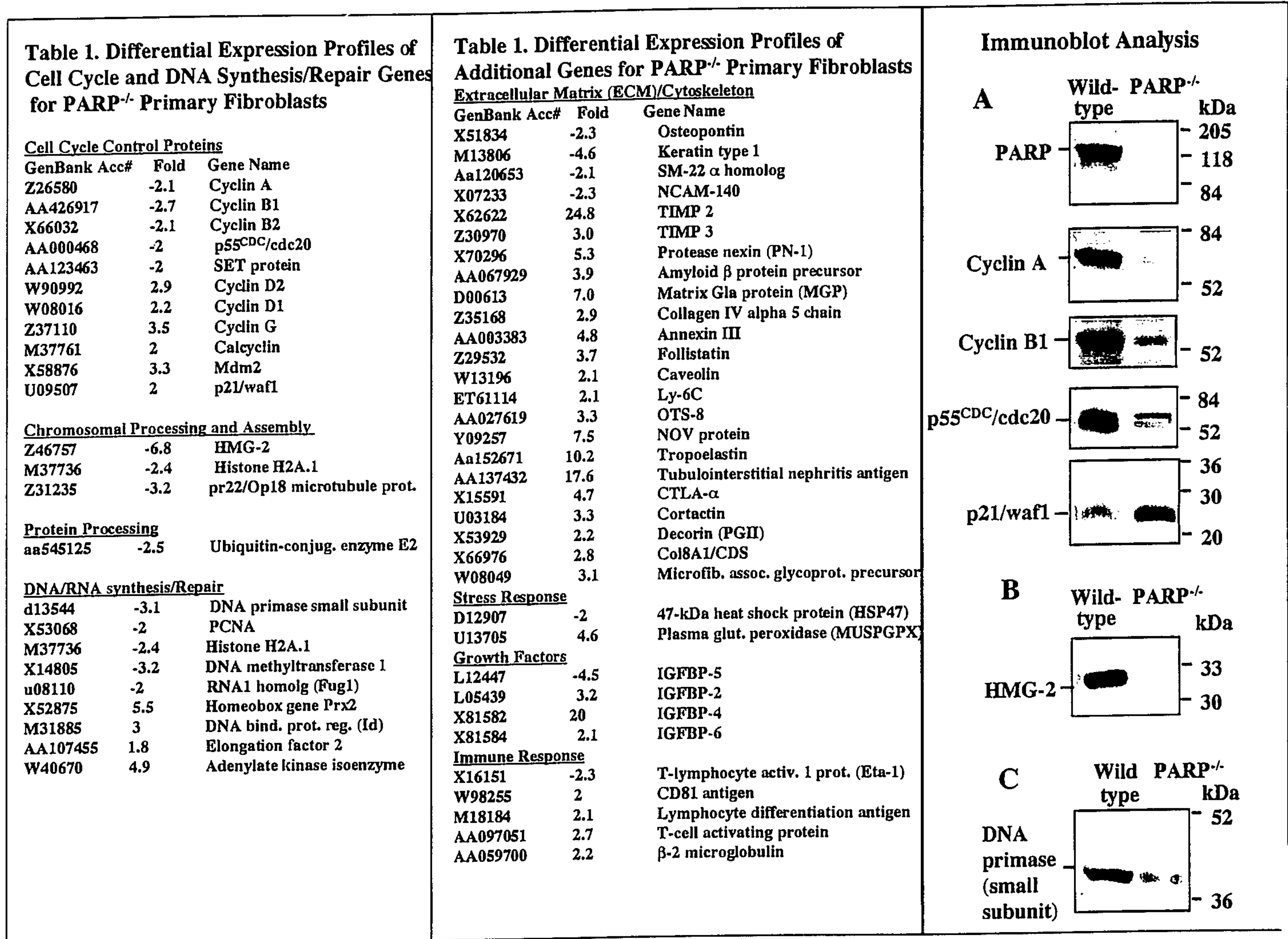

Table 1. Differential Expression Profiles of Cell Cycle and DNA Synthesis/Repair Genes for PARP$^{-/-}$ Primary Fibroblasts

GenBank Acc#	Fold	Gene Name
Cell Cycle Control Proteins		
Z26580	-2.1	Cyclin A
AA426917	-2.7	Cyclin B1
X66032	-2.1	Cyclin B2
AA000468	-2	p55^{CDC}/cdc20
AA123463	-2	SET protein
W90992	2.9	Cyclin D2
W08016	2.2	Cyclin D1
Z37110	3.5	Cyclin G
M37761	2	Calcyclin
X58876	3.3	Mdm2
U09507	2	p21/waf1
Chromosomal Processing and Assembly		
Z46757	-6.8	HMG-2
M37736	-2.4	Histone H2A.1
Z31235	-3.2	pr22/Op18 microtubule prot.
Protein Processing		
aa545125	-2.5	Ubiquitin-conjug. enzyme E2
DNA/RNA synthesis/Repair		
d13544	-3.1	DNA primase small subunit
X53068	-2	PCNA
M37736	-2.4	Histone H2A.1
X14805	-3.2	DNA methyltransferase 1
u08110	-2	RNA1 homolg (Fug1)
X52875	5.5	Homeobox gene Prx2
M31885	3	DNA bind. prot. reg. (Id)
AA107455	1.8	Elongation factor 2
W40670	4.9	Adenylate kinase isoenzyme

Table 1. Differential Expression Profiles of Additional Genes for PARP$^{-/-}$ Primary Fibroblasts

GenBank Acc#	Fold	Gene Name
Extracellular Matrix (ECM)/Cytoskeleton		
X51834	-2.3	Osteopontin
M13806	-4.6	Keratin type 1
Aa120653	-2.1	SM-22 α homolog
X07233	-2.3	NCAM-140
X62622	24.8	TIMP 2
Z30970	3.0	TIMP 3
X70296	5.3	Protease nexin (PN-1)
AA067929	3.9	Amyloid β protein precursor
D00613	7.0	Matrix Gla protein (MGP)
Z35168	2.9	Collagen IV alpha 5 chain
AA003383	4.8	Annexin III
Z29532	3.7	Follistatin
W13196	2.1	Caveolin
ET61114	2.1	Ly-6C
AA027619	3.3	OTS-8
Y09257	7.5	NOV protein
Aa152671	10.2	Tropoelastin
AA137432	17.6	Tubulointerstitial nephritis antigen
X15591	4.7	CTLA-α
U03184	3.3	Cortactin
X53929	2.2	Decorin (PGII)
X66976	2.8	Col8A1/CDS
W08049	3.1	Microfib. assoc. glycoprot. precursor
Stress Response		
D12907	-2	47-kDa heat shock protein (HSP47)
U13705	4.6	Plasma glut. peroxidase (MUSPGPX)
Growth Factors		
L12447	-4.5	IGFBP-5
L05439	3.2	IGFBP-2
X81582	20	IGFBP-4
X81584	2.1	IGFBP-6
Immune Response		
X16151	-2.3	T-lymphocyte activ. 1 prot. (Eta-1)
W98255	2	CD81 antigen
M18184	2.1	Lymphocyte differentiation antigen
AA097051	2.7	T-cell activating protein
AA059700	2.2	β-2 microglobulin

Figure 28 (Table 1, 2)

As noted, we view the back up of Western analysis to be critical controls for the proposed JP-8 project, as will be emphasized by array data with p21 and PCNA expression below. Cyclins associate with and regulate cyclin-dependent kinases (cdk) that subsequently phosphorylate specific targets whose activities are critical to cell cycle progression. Whereas cyclin A is a critical cell cycle regulator required for DNA synthesis during S through G_2 phase as well as for mitosis, cyclin B1 is a key regulator of mitosis and comprise the regulatory subunit of the M-phase promoting factor (MPF). Disruption of normal cyclin A expression, as noted in the PARP$^{(-/-)}$ fibroblasts by microarray and immunoblot analysis, causes cells to arrest at G_2 phase, which might partially explain the G_2-M accumulation noted in these cells. *Interestingly,*

PARP or poly(ADP-ribosyl)ation is implicated to play a role at or near the S-G_2 transition in growing cells given that poly(ADP-ribose) levels increase from early to mid-S phase, with a second large peak noted at the S-G_2 transition, and chemical inhibitors of PARP arrest cells at this stage of the cell cycle.

Similar to cyclin A, inhibition of cyclin B1 transcription also prevents G_2-M transition suggesting that the down-regulation of cyclin B noted in PARP-null cells also contributes to the G_2-M accumulation in these cells. *We may speculate that the toxicity of JP-8 may be manifested in similar down-regulations of many of the important cell cycle regulatory proteins, since growth reduction and death are noted.* Furthermore, MPF, which comprises cyclin B and its binding partner cdc2 kinase, regulate mitotic initiation by phosphorylating and activating enzymes implicated in chromatin condensation, nuclear membrane breakdown, and mitosis-specific microtubule re organization are down regulated. This implies a mechanism by which mitotic misregulation by down-regulation of cyclin B1 blocks normal chromosome segregation or cytokinesis during mitosis, thus, contributing to development of tetraploidy and aneuploidy in PARP-deficient cells.

Aside from the cell cycle regulatory genes, a group of proteins involved in spindle assembly and chromosome segregation, processing, and assembly are also down-regulated 2.4 to 6.8 fold in PARP-null cells (Table 1), including histone H2A.1, HMG-2, and pr22 microtubule protein. The significant down-regulation of HMG-2 expression is further confirmed at the protein level by immunoblot analysis (Fig. 28B). Microarray analysis of PARP-deficient cells (Table 1) also show decreased expression of the ubiquitin carrier protein E2, which might be considered a candidate JP-8 regulated gene since it is a component of the ubiquitin proteasome pathway that mediates selective degradation of key regulatory proteins including the cyclins misregulation of this pathway by downregulation of E2 in $PARP^{(-/-)}$ cells can thus also contribute to imprecise and coordination of late mitotic events.

DNA/RNA synthesis and repair genes (also genes that may be expected to show changes due to (**components of JP-8**), including DNA primase small subunit, PCNA and DNA *methyltransferase* were also noted in these explorative studies performed during the later year of the earlier grant. The decreased expression if DNA primase in $PARP^{(-/-)}$ cells is consistent with our previous results showing down-regulation of expression of DNA pol α and DNA primase in PARP-depleted antisense cells during reentry into S-phase. The reduced expression levels for these essential DNA replication/repair proteins may partially explain the slower growth rate and proliferation defects reported in PARP-deficient cells and it should be revealing to examine these series of genes, with respect to JP-8 exposure. Several of the "Tox-chips" contain probes for repair genes which we anticipate using this current year

Interestingly, parallels can be noted between down-regulated genes in $PARP^{(-/-)}$ cells and alterations of gene expression that occur in replicative senescence or those associated with normal or premature aging, as determined by our Scripps Institute collaborators. Interestingly, down-regulation of PARP is among the notable changes in gene expression that we recently reported to be associated with normal or premature

aging (59), implying that decrease in PARP expression may be an early factor in the agin process (table 1, 2).

Although $PARP^{(-/-)}$ mice have not been shown to be cancer-prone or to have shorter life spans, these mice exhibit extreme sensitivity to γ-irradiation and genotoxic agents and increased genomic instability. Fifty known genes and 12 ESTs were identified to be up regulated in primary $PARP^{(-/-)}$ fibroblasts. The wide range of genes that are induced in PARP-null cells appear to show correlation with cancer progression, senescence, or age-related diseases. A significant number of induced genes (22/50) encode extracellular matrix components or cytoskeletal proteins, such as Collagen IV, Annexin III, caveolin, cortactin, and keratin 1, which are upregulated ~2 to 7 fold (Table 1). Cortactin, which is up-regulated in PARP null cells, is implicated in signaling pathways of mitogenic receptors and adhesion molecules mediating actin cytoskeletal reorganization and is commonly amplified and overexpressed in several human cancers, inducing their invasive or metastatic properties by promoting formation of invadopodia during tumor cell migration and invasion. [Altogether, these same types of parallels between expression of genes altered by JP-8 exposure and known markers of cancer progression and aging, **if observed in our current 3 year grant**, it may imply that some features of a variety of humantoxic phenomena may result at least in part from alterations of gene expression due this agent.

Overexpression of ECM proteins is a hallmark of replicative senescence, which has been associated with changes in gene expression that contribute to pathogenesis of cancer and age-related diseases, as has been noted in the gene expression profile of PARP deficient cells (Table 1) as well as during normal of premature aging (59). Interestingly, the products of a number of genes induced by PARP deficiency have also been implicated in age-related diseases. For example, PARP deficiency induces Alzheimer's β-amyloid precursor protein (APP), which has also been shown to increase expression in senescent cells (86), as well as during human aging.

Accordingly, we are currently experimentally attacking JP-8 toxic exposure by DNA array in essentially the same manner as we have successfully addressed aging and PARP K/O. Undoubtedly, genetic techniques may change dramatically over the 3 year Renewal period. There is no doubt that we are fortunate that our collaborator, Dr. Peter Schultz, on this project (as noted above) is involved in the most advanced and cutting edge advancements in this new technology. Our laboratory will provide a viable and informative genotoxicological program in this area.

RECENT PUBLICATIONS
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3.0 YEARS (4/98-6/01)

Rosenthal, D. S., Simbulan-Rosenthal, C. M., Liu, W.F., Stoica, B., and **Smulson, M.E.** Mechanisms of JP8 jet fuel toxicity II: induction of necrosis in skin fibroblasts and keratinocytes and modulation of levels of Bcl-2 family members. Toxicol. Applied Pharmacol. 171:107-116 (2001).

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Simbulan-Rosenthal, C., Rosenthal, D. S., Iyer, S., Boulares, H., and **Smulson, M.E.** Requirement for a transient poly(ADP-ribosyl)ation of p53 and other nuclear proteins at the early stages of apoptosis. Proc. American Association for Cancer Research 40: 222, 90th Annual Meeting, Philadelphia, PA (1999).

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CURRICULUM VITAE

NAME	POSITION TITLE
MARK E. SMULSON	Professor of Biochemistry and Molecular Biology and Radiation Biology (Joint Appointment), Co-Director Radiation Biology Program, Lombardi Cancer Center

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Washington & Lee University, Lexington, VA	A.B.	1958	Chemistry
Cornell University, Ithaca, NY	M.S.	1961	Nutrition
Cornell University, NY	Ph.D.	1964	Biochemistry
Einstein Medical Center, Philadelphia, PA	Post-doc	1965-65	Nucleoside Analogs
NIH, Bethesda, MD	Post-doc	1966-67	Tumor Molecular Biology

PROFESSIONAL EXPERIENCE

1967-1972; 1972-1978 Assistant Professor; Associate Professor of Biochemistry, Georgetown University Medical School, Professor of Biochemistry

1978 - present Professor of Biochemistry and Molecular Biology Georgetown University

1989 - present Lombardi Cancer Center, Co-Director, Program of Radiation Biology

SPECIAL RECOGNITION

- Awarded First Annual Dean's Prize for Biomedical Research, 1988
- Meeting Organizer International Meeting on Poly(ADP-Rib), 6/73 at NIH.
- Organizer Princess Takamatsu Cancer Meeting, Tokyo, Japan (9/82).
- Organizer, session of Winter Gordon Conference on DNA repair, February 1991.

AUTHORSHIP OF BOOKS AND MONOGRAPHS

Smulson, M.E. and Sugimura, T.,eds, *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Developments in Cell Biology*, Vol. 6, Elsevier/North Holland, 1980.

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