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13. ABSTRACT (Maximum 200 words)

Poly(ADP-ribose) polymerase (PADPRP) is a chromatin-bound enzyme which is pivotal in cellular recovery from DNA strand break damage. PADPRP requires DNA for activity; it is significant that the catalytic activity of this enzyme is directly coordinated with the number of DNA strand breaks in DNA, both in vitro as well as in vivo. Thus, poly(ADP-ribosylation) is rapidly modulated in response to environmentally significant DNA-damaging agents; this probably represents the most initial response of the cell to genotoxic damage to the genome.

One of the major aims over the past few years of this project has been to establish and characterize cells stably transfected with PADPRP antisense cDNA under the control of an inducible promoter and to establish conditions under which significant depletion of nuclear PADPRP could be achieved. This approach has been particularly successful in assessing the potential roles of poly(ADP-ribosylation) in a variety of biological processes, all involving DNA strand breaks, without the use of non-specific chemical inhibitors. Thus, we have assigned biochemical roles for PADPRP in the recovery of cells with exposure to mutagenic agents, gene amplification and DNA replication.

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ANNUAL TECHNICAL REPORT

**THE KEY INVOLVEMENT OF POLY(ADP-RIBOSYLATION) IN DEFENSE
AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES**

October 12, 1994

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A. Summary of Previous Aims

AIM I: Expression of "analog" PADPRPs in mammalian cells: role and mechanism of PADPRP in DNA repair.

(1) Pilot Approaches Directed at Expression of PADPRP in *E. coli*; (2) Expression of *E. coli* Synthesized PADPRP; (3) Bacterially Expressed Deletion in Active Site Domain; (4) Preliminary Expression in *E. coli* of altered DNA Binding Domain Enzyme (Finger Swap between PADPRP and GR); (5) Expression in *E. coli* of GR DNA Binding Substituted PADPRP.

AIM II: Cytotoxicity and DNA repair studies in stably transfected cells with altered potential for poly ADP-ribosylation - effects of environmental toxic agents.

(1) Methods for DNA Repair Measurements in Stably Transfected cells; (2) Cytotoxicity and Survival Measurements for Cells in the Presence and Absence of Environmental Toxic Agents; (3) Fluorescent Activated Cell Sorter Analysis.

B. Status of Progress Towards Research Objectives

Progress During Year 2 on Aim I: (Specific Aim I: THE MECHANISM OF PADPRP IN DNA *IN VITRO* REPAIR - DELETION MUTANTS OF PADPRP)

Cherney, B. W., Chaudhry, B., Bhatia, K., Butt, T.R., and Smulson, M.: Expression and Mutagenesis of Human Poly(ADP-Ribose) Polymerase as a Ubiquitin Fusion Protein from *Escherichia coli*. Biochemistry. **30**:10420-10427. (1991).

- Expression of PADPRP as a fusion protein in *Escherichia coli*. In brief, 5 major deletion mutants encompassing the 3 functional domains of PADPRP were expressed in *E. coli*. The full-length, non-deleted bacterially expressed PADPRP had catalytic properties which were nearly identical to that of PADPRP.

We have utilized information gained in this study to overexpress in bacteria, in pure form, the PADPRP-DNA binding domain (DBD) in order to use the unique and strong DNA strand break binding property of this protein to begin to develop a Biomarker assay for genotoxicity. Additionally, the deletion mutants of PADPRP were experimentally useful to test a new model for PADPRP's direct role in binding and protecting DNA strand breaks(SBs).

Smulson, M., Istock, N., Ding, R., and Cherney, B.: Deletion Mutants of Poly(ADP-Ribose) Polymerase Support A Model of Cyclic Association and Dissociation of Enzyme from DNA Ends During DNA Repair. Biochemistry. **33**(20):6186-6191. (1994).

We tested whether PADPRP cycles between an unmodified form, which protects DNA breaks, and a poly(ADP-ribosyl)ated form which is released from DNA, thereby allowing access to repair enzymes; using the above mutants comprising deletions in the three major functional domains of PADPRP, deletion mutants (Tables 1 and 2) with an intact NH2-terminal DNA-binding domain, and therefore capable of binding to DNA SBs in the *in vitro* assay, inhibited repair; however, whether the deletion was in the NAD-binding active site domain or the automodification domain, the inhibition of repair exerted by these mutant proteins was not alleviated by NAD. PADPRP with a deletion in the DNA-binding domain did not inhibit a repair. Thus, the

behavior of these analogs proved to be consistent with a model proposed by Lindahl. Also, exposure of the nuclei from mid-S phase HeLa cells to NAD increased single deoxynucleotide incorporation as catalyzed by endogenous DNA polymerase.

Table 1.

Effect of PADPRP deletion mutants on DNA repair *in vitro*.

extract	supplement	DNA repair (%)	
		-NAD	+NAD
complete HeLa cell		13.7 ± 0.28	33.0 ± 0.35
PADPRP-depleted HeLa cell		45.3 ± 3.74	44.3 ± 2.43
PADPRP-depleted HeLa cell	PADPRP	8.7 ± 2.41	22.0 ± 3.8
PADPRP-depleted HeLa cell	TM 1	27.3 ± 2.29	26.4 ± 2.67
PADPRP-depleted HeLa cell	TM 4	18.9 ± 0.62	19.3 ± 4.25
PADPRP-depleted HeLa cell	TM 5	42.5 ± 2.20	42.3 ± 0.86
PADPRP-depleted HeLa cell	TM 6	31.6 ± 6.52	30.3 ± 5.94

Table 2.

NAD does not affect DNA repair in nuclear extracts derived from cells induced to express PADPRP antisense mRNA

Antisense Induction (cell extract)	DNA Repair (%)	
	-NAD	+NAD
-	13.9	20.8
+	21.5	24.6

Rosenthal, D., Hong, T., Cherney, B., Zhang, S., Shima, T., Danielsen, M., and Smulson, M. Expression and characterization of a fusion protein between the catalytic domain of poly(ADP-ribose) polymerase and the DNA binding domain of the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* 202(2):880-887. (1994).

A fusion protein comprising the DNA-binding region of the glucocorticoid receptor and the catalytic domain of poly(ADP-ribose) polymerase was constructed as proposed in AIM I of the present AFOSR period. This chimeric protein was expressed both in *E. coli* and in eukaryotic cells, and was recognized by antibodies to both polymerase and the glucocorticoid receptor. Similar to polymerase, the chimera produced bona-fide poly(ADP-ribose) polymers covalently bound to protein, and was inhibited by 3-aminobenzamide. Like the authentic glucocorticoid receptor, the fusion protein formed a stable complex with DNA containing the glucocorticoid response element. In mammalian cells, the fusion protein significantly and specifically inhibited the ability of the glucocorticoid receptor to stimulate a reporter construct. These results indicate that polymerase activity can be targeted to specific DNA sequences and modulate gene expression.

Progress During Year 2 on Aim I: (Specific Aim II: ELUCIDATION OF THE BIOLOGICAL FUNCTION(S) OF PADPRP BY THE EXPRESSION OF ANTISENSE PADPRP TRANSCRIPTS IN HELA AND 3T3-L1 CELLS)

One major aim of this project was to establish and characterize stably transfected cells with antisense cDNA to PADPRP driven by an inducible promoter and to establish conditions where depletion of PADPRP could be achieved, only upon induction. These cell would be available to assess the role of poly(ADP-ribosyl)ation DNA repair and in recovery from genotoxic agents (Table 1) without using non-specific chemical inhibitors. We have been successful with this approach and, during the past year several studies have been published describing the initial properties of these cell lines.

Ding, R., Pommier, Y., Kang, V.H., and Smulson, M.: Depletion of Poly(ADP-ribose) Polymerase by Antisense RNA Expression Results in a Delay in DNA Strand Break Rejoining. *J. Biol. Chem.* 267(18):12804-12812. (1992).

A recombinant expression plasmid was prepared with the MMTV promoter upstream of the antisense-oriented PADPRP cDNA. Expression of the antisense RNA was under strict control, with negligible effects on cell growth being apparent in the absence of inducer. Consistent with the previously described stability of PADPRP ($T^{1/2}$ of at least 2 days, *in vivo*), 48 to 72 hr were required after induction of antisense RNA expression by Dex for the highly abundant cellular concentration of PADPRP, to be reduced by > 80% (Fig 1).

The depletion of endogenous PADPRP, as mediated by induced antisense RNA expression, was established. The chromatin of PADPRP-depleted cells was shown to have an altered structure as assessed by DNase I susceptibility. Most significant with respect to the Aims of this proposal was the observation that **cells depleted of PADPRP were not able to commence DNA repair of alkylating agent-damaged DNA** (Fig 2). However, DNA repair capacity was reestablished at later time periods, indicating that PADPRP may contribute to alterations in chromatin structure that occur initially in DNA SB rejoining, and that the concentration of the enzyme in nuclei exceeds the requirement for DNA repair/replication.

Figure 1

Effect of Dex induction of antisense RNA on cellular PADPRP content as determined by immunostaining



A Absence Dexamethasone B Presence Dexamethasone

Figure 2

The repair of MMS-induced SSBs in control and antisense cells

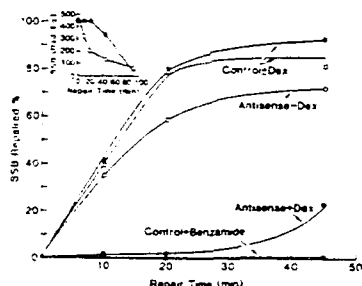
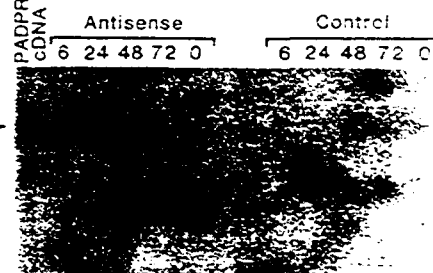


Figure 3

Expression and stability of PADPRP antisense transcripts in PADPRP - as[7] and control cell lines after induction by dexamethasone

Dexamethasone Treatment (Hour)



The success of this technique with respect to lowering the poly ADPR response subsequent to strand breaks forms a basis to explore the role of this signal in DNA repair and cell survival in differing types of cells and tissues which may be at risk for exposure to environmental hazards.

Ding, R. and Smulson, M.: Depletion of Nuclear Poly(ADP-Ribose) Polymerase by Antisense RNA Expression: Influences on Genomic Stability, Chromatin Organization and Carcinogen Cytotoxicity. Cancer Research. 54:4627-4634. (1994).

We next utilized the ability of antisense expression in HeLa cells to deplete cellular levels of the enzyme to investigate several other pleiotropic effects of PADPRP in strand break rejoining reactions. It was shown that: 1) Gene amplification was increased 2-3 fold in cells depleted of PADPRP compared to controls. 2) Chromatin structure was significantly altered in PADPRP-depleted cells, as indicated by reduced initiation and elongation of poly(ADP-ribose) chains attached to various nuclear protein acceptors (Fig. 4), altered poly ADPR of histone H1, and an increased susceptibility to micrococcal nuclease digestion. *These latter*

biochemical properties of PADPRP have been continually studied throughout the entire course of this project.

3) The survival of PADPRP-depleted antisense cells exposed to the DNA alkylating agent methyl methane sulfonate and to nitrogen mustard was significantly reduced, relative to that of control cells (Fig. 5).

Figure 4.

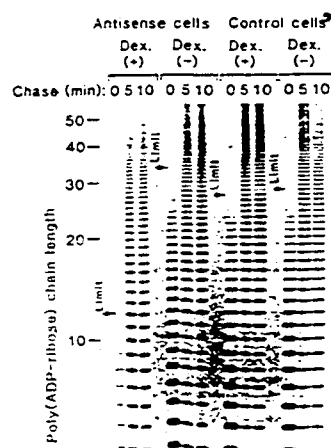


Figure 5.

Effect of PADPRP antisense RNA expression on survival of nitrogen mustard-treated HeLa Cells.

Nitrogen mustard (μM)	Cell Survival (%)			
	PADPRP-as[20]		Control Cells	
	Dex.(+)	Dex.(-)	Dex.(+)	Dex.(-)
5	18.3 ± 5.03	41.2 ± 8.5	28.7 ± 1.7	28.4 ± 2.0
10	0.42 ± 0.075	3.74 ± 0.84	3.53 ± 0.15	2.83 ± 0.15
20	0.062 ± 0.023	0.47 ± 0.11	0.38 ± 0.07	0.48 ± 0.01

* mean $\pm$ SD

Ding, R., Stevnsner, T., Bohr, V. and Smulson M. Effect of Depletion of Poly(ADP-ribose) Polymerase on Gene specific DNA Repair of Pyrimidine Dimers and Alkylation damage. Nucleic Acid Research. In Press. (1995).

Repair of alkylation adducts in DHFR gene caused by Nitrogen mustard (HN2) treatment - In AIM II we also proposed to establish this assay, to be used to test hazardous compounds. Thus, in order to follow through on the above observations and better describe the role of PADPRP in gene-specific repair, especially in preferential repair of active gene damage introduced by HN2, we analyzed the repair of the gene for DHFR in induced antisense cells were determined for DHFR gene in collaboration with Vil Bohr (NCI) using Southern hybridization analysis which was developed by Bohr while in the Hanawalt lab.

Figure 6

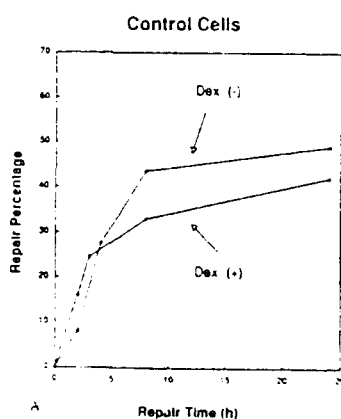
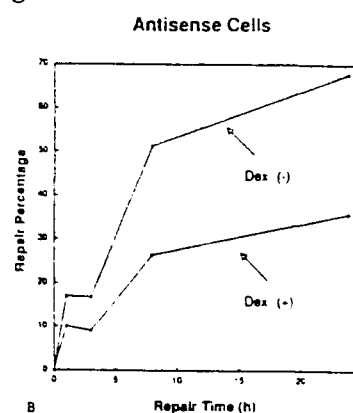


Figure 7



Preferential repair of DHFR gene

Figs. 6 and 7 show that induced antisense cells remove about 20 – 30% of alkylation adducts within 8 and 24h, respectively. However, the HN2-induced lesions were removed more efficiently within a 24 h period in the antisense cells with no Dex treatment. Thus, about 50% and 70% of repair occurred within 8 h and 24

h, respectively. The apparent distinction between repair patterns of induced and non-induced antisense cells indicates that an 80% reduction in PADPRP by antisense induction significantly inhibits the repair of HN2-induced DNA lesions in the active DHFR gene.

Smulson, M.E., Kang, V.H., Ntambi, J.M., Rosenthal, D.S., Ding, R., and Simbulan, C.M.G. Requirement for the Expression of Poly (ADP-ribose) Polymerase During the Early Stages of Differentiation of 3T3-L1 Preadipocytes, as Studied by Antisense RNA Induction. J. Biol. Chem. In Press. (1995).

A characterized stably transfected cell line of 3T3-L1, pre-adipocytes was also established where PADPRP levels were also manipulated by expression of antisense mRNA. Key observations made with this system were that PADPRP levels significantly increase, by both Western and transcript analysis, during the first day of the differentiation and then progressively decrease during the course of differentiation.

- **Reduced PADPRP and Preadipocyte Initiation of DNA Replication** - In contrast, PADPRP levels do not increase during differentiation in antisense induced cells. Nor do the cells undergo the normal 2-3 rounds of cell division, required during the initial 1-2 days of differentiation induction. The antisense cells occasionally progressed through one round of cell division; however, this resulted in extensive cytotoxicity and the cells proceeded to **apoptosis**, perhaps as a result of Okazaki fragments which are not able to be ligated and hence accumulate.

C. Technical Journal Publications

Publications Published Since Last Technical Report:

Ding, R., Stevnsner, T., Bohr, V. and **Smulson M.** Effect of Depletion of Poly(ADP-ribose) Polymerase on Gene specific DNA Repair of Pyrimidine Dimers and Alkylation damage. Nucleic Acid Research. In Press. (1995).

Smulson, M.E., Kang, V.H., Ntambi, J.M., Rosenthal, D.S., Ding, R., and Simbulan, C.M.G. Requirement for the Expression of Poly (ADP-ribose) Polymerase During the Early Stages of Differentiation of 3T3-L1 Preadipocytes, as Studied by Antisense RNA Induction. J. Biol. Chem. In Press. (1995).

Smulson, M.E. Poly(ADP-ribose) Polymerase Gene on Chromosome 1q: Early Role in Differentiation Linked Replication; Gene on Human Chromosome 13q: Marker of Carcinogenesis. Molecular and Cellular Biochemistry. In Press. (1994).

Ding, R., and **Smulson, M.** Depletion of Nuclear Poly(ADP-Ribose) Polymerase by Antisense RNA Expression: Influences on Genomic Stability, Chromatin Organization and Carcinogen Cytotoxicity. Cancer Research. 54:4627-4634. (1994).

Avila, M.A., Velasco, J.A., **Smulson, M.E.,** Dritschilo, A., Castro, R., and Notario, V. Functional expression of human poly(ADP-ribose) polymerase in *Schizosaccharomyces pombe* results in mitotic delay at G1, increased mutation rate, and sensitization to radiation. Yeast. 10:1003-1017. (1994).

Rosenthal, D., Hong, T., Cherney, B., Zhang, S., Shima, T., Danielsen, M., and **Smulson, M.** Expression and characterization of a fusion protein between the catalytic domain of poly(ADP-ribose) polymerase and the DNA binding domain of the glucocorticoid receptor. Bioch. Biophys. Res. Commun. 202(2):880-887. (1994).

Smulson, M., Istock, N., Ding, R., and Cherney, B. Deletion Mutants of Poly(ADP-Ribose) Polymerase Support A Model of Cyclic Association and Dissociation of Enzyme from DNA Ends During DNA Repair. Biochemistry. 33:(20)6186-6191. (1994).

Su, Y., Rosenthal, D., **Smulson, M.**, and Speigel, S. Sphingosine-1-Phosphate, A Novel Signaling Molecule, Stimulates DNA-Binding Activity of AP-1 in Quiescent Swiss 3T3 Fibroblasts. J. Biol. Chem. 269(23):16512-16517. (1994).

Pero, R., Berglund, G., Christie, N.T., Cosma, G.N., Frenkel, K., Garte, S.J., Jacobson, E.L., Jacobson, M.K., Janzon, L., Seidegård, J., **Smulson, M.E.**, and Troll, W. The Malmö Diet and Cancer Study: The Biomarker Program. Journal of Internal Medicine. 233:69-74. (1993).

Lyn, D., Deaven, L.L., Istock, N., and **Smulson, M.** The Polymorphic Poly(ADP-ribose) Polymerase Gene in Humans Interrupts an Endogenous *Pol*-like Element on 13q34. Genomics. 18:206-211. (1993).

Lyn, D., Cherney, B., Lalande, M., Berenson, J., Lupold, S., Bhatia, K., and **Smulson, M.** A duplicated region is responsible for the poly(ADP-ribose) polymerase polymorphism on chromosome 13 associated with a predisposition to cancer. Am. J. Hum. Genetics. 52:124-134. (1993).

Publications Submitted and in Preparation

Lyn, D, Istock, N.L., **Smulson, M.** Conservation of Sequences of between Human and Gorilla Lineages: The ADP-Ribosyltransferase (NAD⁺) Pseudogene 1 and the Neighboring Retroposons. Gene. Manuscript submitted. (1994).

Ding, R., Rosenthal, D., Cherney, B., and **M. Smulson.** PolyADP-ribose Polymerase DNA Binding Domain: A Sensitive Biotinylated Probe for Detection of Apoptosis. (1994).

Ding, R., Jackman, J., **Smulson, M.** Depletion of PolyADPRP by Antisense Induction Enhances to p53 to DNA Breaks. (1994).

D. Professional Personnel

No changes to the professional staff.

E. Interactions

1. Papers Presented at Meetings, Conferences and Seminars:

M.E. Smulson. "ADP-ribosylation Science Is Now Emerging as an Important Field in Cancer Research. The 11th International Symposium on ADP-ribosylation DNA Repair. Signal Transduction. Strasbourg, Bischenberg, France, September 17 - 21, 1994.

2. Consultative Functions to Other Laboratories and Agencies:

Smulson, M.E. Molecular Biology Basis for the Response of Poly (ADP-rib) Polymerase and NAD Metabolism to DNA Damage Caused by Mustard Alkylating Agents. Contract No.: DAMD17-90-C-0053. Duration: 4/30/90-9/29/94.

F. New Discoveries, Inventions, Patents

Not applicable.

G. Additional Insight

PADPRP requires DNA for activity; a major clue toward ascertaining the function of the enzyme has been the observation described by Benjamin and Gill that the *catalytic activity of PADPRP is directly coordinated to the numbers of strand breaks in DNA*. Thus, relevant to the objectives of AFOSR is the fact that PADPRP causes the poly ADP-ribosylation of nucleosomal proteins in response to a variety of DNA-damaging agents including environmentally toxic agents such as alkylating compounds, aromatic hydrocarbons, UV light and ionizing irradiations.

In **AIM I** of the renewal program, we propose to exploit the experimental protocols established during the last granting period with HeLa cells transfected with PADPRP antisense RNA constructs to further study the role of this enzyme in DNA repair, strand rejoining reactions, and chromatin reassembly in other cell systems, appropriate to environmental agents of interest to AFOSR (*i.e. ADN, hydrazine, trichloroethylene, tetrochloroethylene, N-Nitrosodimethylamine, and cadmium, etc.*). For example, a novel human immortalized karytinocyte cell line transfected with antisense to PADPRP has been developed in pilot studies, since skin is one tissue at high risk to exposure to a number of environmental agents. These engineered skin tissue culture cells have been grafted onto mice and form a layer of human skin, which is histologically identical to normal skin, then, using new **Biomarker** methods, piloted in new **AIM II**, we have shown that topical addition of an alkylating agent (as a prototype for other agents) elicits rapid synthesis of poly(ADP-ribose) polymer (an indirect measure of DNA breaks). Other types of cells and organs will be tested in new **AIM I**. We will also determine whether sufficient PADPRP-DNA-binding domain can also be induced (in various cells) as an alternative method to inhibit endogenous nuclear polymerase activity completely.

The experiments projected in **AIM II** of our renewal application are a logical extension of the biology and biochemistry information gained in new **AIM I**. These new approaches represent a practical utilization of this information by developing two new **Biomarker** assays for the assessment of environmental hazardous agents. Observations relevant to this aim include the following: (a) The biosynthesis of the unique biopolymer, poly(ADP-ribose) is one of the earliest responses of cells to DNA damage, as elicited by many environmental hazardous agents. (b) The DNA-binding Domain of PADPRP is among the most effective cellular proteins which binds to both single and double strand DNA breaks. Our laboratory, during the present period, has overexpressed this human protein in bacteria. We propose to develop two new **Biomarker** assays, of which preliminary data suggests a high level of feasibility. On the one hand, we will develop a bioassay using the ability of antibody, specific to poly(ADP-ribose) polymer to detect this product (and indirectly DNA damage) on fixed cells or tissues. Secondly, we will develop a similar type of assay to detect DNA strand breaks in tissues cells or tissues by the unique binding of the PADPRP-DBD on fixed slides or tissues which have been exposed to hazardous agents and subsequently the detection of this bound protein only to DNA ends by immunological procedures. The objectives of new **AIM II** should allow the possibility of **Technology Transfer** to the AF based upon knowledge gained during the earlier granting periods of this project. It also offers a logical collaboration between our extramural program with intramural programs within the AFOSR. One such collaboration has already been initiated between our laboratory and that of Lt. Col. Jay Kidney, Toxicology Division, Armstrong Laboratory, Wright-Patterson AFB, utilizing an *in vitro* liver slide method, ongoing in his laboratory.