[CANCER RESEARCH 56, 2715–2717, June 15, 1996]

Advances in Brief

Trans-Dominant Inhibition of Poly(ADP-ribosyl)ation Potentiates Carcinogen-induced Gene Amplification in SV40-transformed Chinese Hamster Cells[1]

Jan-Heiner Küpper,[2] Marcus Müller, and Alexander Bürkle

Deutsches Krebsforschungszentrum, Forschungsschwerpunkt Angewandte Tumorvirologie, Abteilung 0610, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

Abstract

Poly(ADP-ribose) polymerase (PARP) is an evolutionally conserved nuclear protein present in most eukaryotic species and catalyzes the formation of ADP-ribose polymers covalently attached to proteins. PARP is strongly activated by DNA single- or double-strand breaks and is thought to be involved in cellular responses to DNA damage. Based on the SV40-transformed Chinese hamster cell line CO60, we had established stable transfectants that overexpress the PARP DNA-binding domain conditionally. DNA-binding domain overexpression led to *trans*-dominant inhibition of poly(ADP-ribosyl)ation and sensitized the cells to genotoxic agents. Using the amplification of chromosomally integrated SV40 DNA as an indicator system, we show here that *trans*-dominant PARP inhibition potentiates genetic instability induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of cells.

Introduction

Poly(ADP-ribosyl)ation of proteins is catalyzed by the nuclear enzyme PARP[3] (EC 2.4.2.30), with NAD^+ serving as substrate, and represents an immediate-early response of most eukaryotic cells to DNA damage (reviewed in Ref. 1). PARP binds to DNA strand breaks by its NH_2-terminal DNA-binding domain, resulting in the activation of its catalytic function.

Although the precise biological role of PARP is not known, this enzyme activity has been linked with several cellular responses to DNA damage, *i.e.*, DNA repair, genetic recombination, and most recently with apoptosis (2–4). It was postulated from different experimental systems that stimulation of the poly(ADP-ribosyl)ation pathway in carcinogen-treated cells counteracts the phenomenon of genetic instability. One form of genetic instability is the amplification of cellular DNA sequences, *e.g.*, oncogenes or drug resistance genes, which is thought to play an important role in carcinogenesis (reviewed in Ref. 5). We had shown previously that the chemical ADP-ribosylation inhibitor 3AB potentiates SV40 DNA amplification induced by the alkylating agent MNNG in the SV40-transformed hamster cell line CO60 (6). Likewise, MNNG-induced methotrexate resistance and amplification of the *dihydrofolate reductase* gene in CHO cells was potentiated by ADP-ribosylation inhibitors (7). Given the accumulating evidence of nonspecific inhibitor effects, we developed the stably transfected CO60 subline COM3, in which inhibition of poly(ADP-ribosyl)ation is achieved by Dex-inducible overexpression of the mere DNA-binding domain of PARP, acting as a dominant-negative mutant of this enzyme (8). Here we demonstrate that MNNG-induced SV40 DNA amplification is potentiated when this cell line is pretreated with Dex to overexpress the DNA-binding domain of PARP.

Materials and Methods

Cell Culture and MNNG Treatment. COM3 and COR2 cells are stable transfectants of the SV40-transformed hamster cell line CO60 and were maintained as monolayers exactly as described (8). Exponentially growing cells were trypsinized and plated at 10^5 (MNNG treatments) or 10^4 (controls) cells per 10-cm Petri dish. One day later, fresh medium supplemented or not with 5×10^{-8} M Dex was given. Dex treatment was performed for 24 h. Thereafter, medium was replaced with medium containing 50 μM MNNG (10 mM stock solution in PBS) or with normal medium. Cells were further incubated in these media under routine conditions for 4 days. In some experiments, 2 mM 3AB (Sigma Chemical Co., München, Germany) was added to the cells 2 h before MNNG treatment and was then continuously present until cells were harvested for DNA extraction.

DNA Amplification Assay. Adherent cells were trypsinized and combined with nonadherent cells. Total cellular DNA was extracted, denatured, and neutralized exactly as described (9). DNA from 5×10^4 cells was blotted onto duplicate nitrocellulose membranes using a dot blot apparatus (Schleicher & Schüll, Dassel, Germany). Blots were dried for 30 min at 80°C and subjected to prehybridization and hybridization in 50% formamide at 42°C as described (6). For the detection of amplified SV40 DNA, the ^{32}P-labeled (10) 2.2-kb *Bam*HI-*Taq*I fragment of SV40 was used while detection of the single-copy *albumin* gene was performed on a parallel blot with the ^{32}P-labeled 700-bp fragment spanning the region between the *Hin*dIII site and the 3′-end of the rat albumin cDNA. Hybridization signals were evaluated by using a phosphorimager (Molecular Dynamics, Krefeld, Germany) or by autoradiography.

To quantitate the SV40 DNA amplification responses to the various treatment conditions, peak area values of the SV40 hybridization signals were divided by the respective albumin values from the parallel blot to correct for the exact amount of DNA loaded. The resulting values were divided by those of untreated control cells, thus yielding "amplification factors."

Results and Discussion

Inhibition of Poly(ADP-ribosyl)ation Potentiates Gene Amplification. By using the overexpressed DNA-binding domain of human PARP as a *trans*-dominant inhibitor of poly(ADP-ribosyl)ation in the stably transfected cell line COM3 (8), we set out to investigate the effect of this type of PARP inhibition on carcinogen-induced DNA amplification. Since COM3 cells were derived from the SV40-transformed Chinese hamster cell line CO60, we chose the amplification of integrated SV40 DNA as a model system, as originally described by Lavi (11). In COM3 cells, the PARP DNA-binding domain expression is controlled by the Dex-inducible mouse mammary tumor virus promoter. COM3 cells also contain an expression construct for the human glucocorticoid receptor facilitating high-level expression of the DNA-binding domain (8). To check for any possible side effects of Dex in combination with glucocorticoid receptor overexpression, we have established CO60 control transfectants overexpressing this receptor but lacking the PARP DNA-binding domain cDNA (8). One of these cell lines, COR2, expresses about twice the amount of glucocorticoid receptor as does COM3 and is competent to amplify

Received 3/11/96; accepted 5/1/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This paper is dedicated to Professor Harald zur Hausen on the occasion of his 60th birthday. Our work was supported by the Deutsche Forschungsgemeinschaft (Bu698/2-2 and 2-3).

[2] To whom requests for reprints should be addressed. Phone: 49 6221 424982; Fax: 49 6221 424962; E-mail: JH.Kuepper@DKFZ-Heidelberg.DE.

[3] The abbreviations are: PARP, poly(ADP-ribose) polymerase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Dex, dexamethasone; 3AB, 3-aminobenzamide; CAD, carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase.

SV40 DNA after MNNG treatment, as holds true for COM3 (see below).

Fig. 1*A* shows representative results of SV40 DNA amplification experiments done in COM3 cells. At 50 μM MNNG, there was about 3-fold amplification of SV40 sequences. In parallel, we observed about 5-fold SV40 DNA amplification in parental CO60 cells (data not shown); thus, amplification amplitudes of COM3 and CO60 cells are comparable. Previously, we had detected about 20-fold SV40 DNA amplification in our CO60 cultures with the same MNNG concentration (6). This difference in the absolute amplitude of SV40 DNA amplification very likely is due to different batches of FCS or different MNNG solvents (DMSO *versus* PBS) used. Pretreatment of COM3 cells with Dex, leading to overproduction of the DNA-binding domain of PARP, caused a drastic potentiation of MNNG-induced SV40 DNA amplification (Fig. 1, *A* and *C*). The MNNG-induced amplification of SV40 DNA sequences was potentiated 6-fold in Dex-pretreated cells compared to untreated cells, whereas there was nearly 2-fold potentiation of amplification in cells that were treated with MNNG plus the chemical PARP inhibitor 3AB. The latter result is in line with our earlier data on CO60 cells showing that under these conditions, 3AB potentiated SV40 DNA amplification about 2.5-fold. To check for any side effects of Dex *per se* in this system, we investigated SV40 amplification in the control cell line COR2 (Fig. 1*B*). MNNG treatment apparently led to a slight increase in SV40 copy number, and this increase was more pronounced in cells co-treated with the chemical PARP inhibitor. However, pretreatment with Dex had no detectable effect on the amplification amplitude in COR2 cells. This was also the case when we tested other MNNG concentrations in the range of 25–100 μM, whereas in COM3 cells, the amplification-potentiating effect of Dex was clearly detectable at all MNNG concentrations tested (data not shown).

We conclude from our results that inhibition of poly(ADP-ribosyl)ation increases genetic instability in carcinogen-treated cells as measured by gene amplification. However, neither DNA-binding domain overexpression nor PARP inhibition with 3AB caused SV40 gene amplification in the absence of DNA damage. This is in contrast to recent data on stably transfected HeLa cells showing increased amplification of the *CAD* gene in the presence of PARP-specific antisense RNA but without any carcinogen treatment of cells (12). To see whether this apparent discrepancy is due to the different amplification systems (SV40 *versus* CAD), we are currently studying the influence of PARP inhibition on *CAD* amplification in COM3 cells.

Interestingly, the effect of *trans*-dominant inhibition of poly(ADP-ribosyl)ation on gene amplification is much more pronounced than the effect exerted by the chemical PARP inhibitor. Immunofluorescence analysis of cellular poly(ADP-ribose) formation revealed that both modalities lead to virtually complete inhibition of polymer formation after treatment with 50 μM MNNG (data not shown). This is in line with the equal efficiency of inhibition we have detected previously by quantitative poly(ADP-ribose) analysis upon 56 Gy of γ-irradiation (8). Thus, there must exist qualitative differences between the two inhibition modalities. It is conceivable that binding of the DNA-binding domain to a DNA strand break is almost irreversible, and indeed, it was shown previously that DNA-binding domain overexpression results in a blockade of MNNG-induced unscheduled DNA synthesis in living cells (13). Comparable results were obtained in an *in vitro* DNA repair system, revealing that unmodified PARP binds tightly to DNA strand breaks and inhibits DNA repair; auto-poly-(ADP-ribosyl)ation of the enzyme then effects its release from the break and renders lesions accessible for DNA repair enzymes (14, 15). In a cell overexpressing the PARP DNA-binding domain, only few DNA strand breaks will be occupied by catalytically active PARP and should, therefore, be protected against such a blockade of DNA repair.

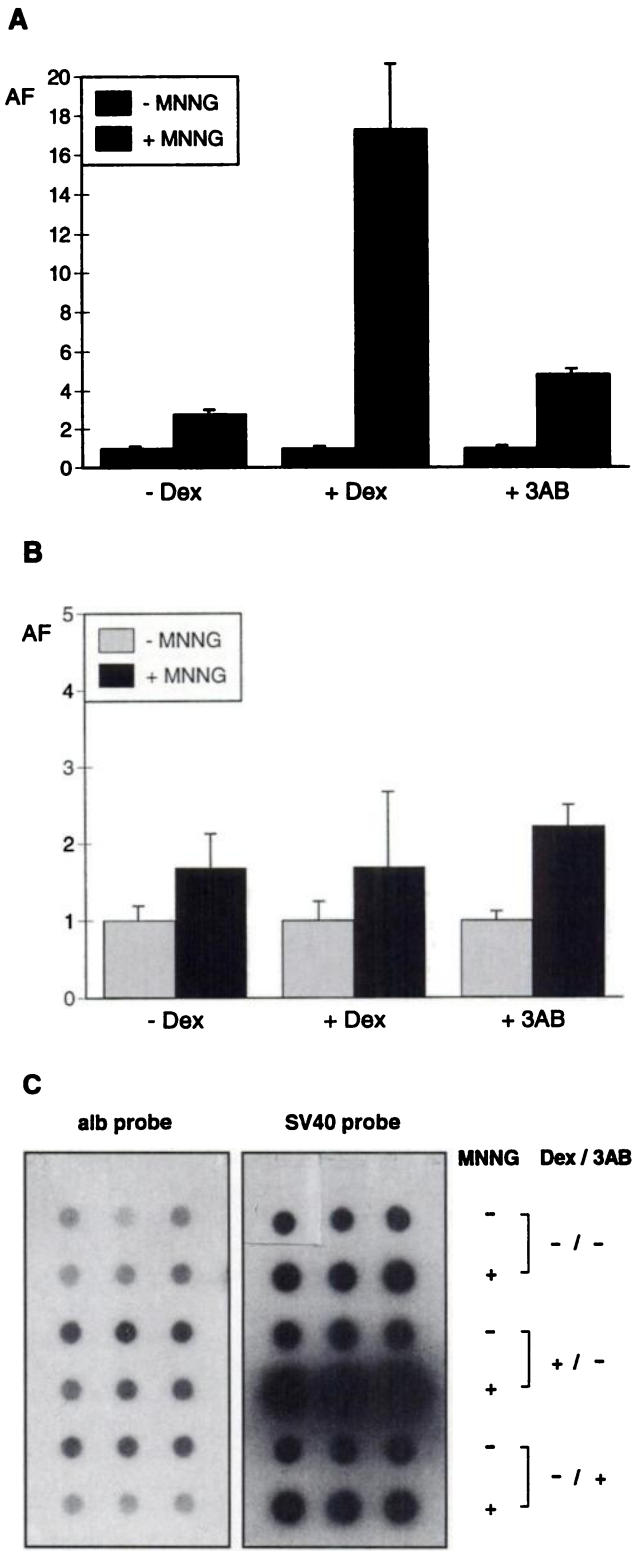

Fig. 1. MNNG-induced SV40 DNA amplification in COM3 and COR2 cells. Treatment of COM3 (*A*) or COR2 cells (*B*) with 5×10^{-8} M Dex, or with 2 mM 3AB, and incubation with 50 μM MNNG were performed as described in "Materials and Methods." Genomic DNA was purified from cells 4 days after carcinogen treatment and subjected to DNA dot blotting. Parallel blots were probed with ^{32}P-labeled SV40 DNA or rat albumin cDNA, respectively. Hybridization signals were evaluated by phosphorimaging and were used to calculate the factors of SV40 DNA amplification (*AF*) in MNNG-treated cells relative to the signals from untreated cells. Hybridization signals of the single-copy *albumin* gene were used to control for the amount of loaded DNA. *A* and *B*, data are the mean values from at least three independent experiments; *bars*, SD. *C*, dot blot autoradiographs from a representative, triplicate SV40 DNA amplification experiment done in COM3 cells. Hybridizations with the albumin and the SV40 DNA probe, respectively, are shown.

By contrast, inhibition of strand break-bound endogenous PARP with benzamide derivatives, acting as competitive inhibitors of the substrate NAD^+, may be less complete; thus, blockades of all of the strand breaks may be weaker, due to low-level PARP automodification still going on. Indeed, it was shown that the temporary presence of even short poly(ADP-ribose) chains on PARP abrogates inhibition of excision repair by this protein (16).

PARP Inhibition and Genetic Instability. MNNG treatment of cells leads to base alkylations that are removed by base-excision repair, in the course of which DNA strand breaks are formed. Depending on the MNNG concentration, the majority of these breaks will be single-strand breaks. Binding of the DNA-binding domain to these breaks inhibits repair and should thus enhance the risk of a second lesion in the neighborhood, which then can lead to DNA double-strand breaks and even chromosomal breaks. Interestingly, it was demonstrated in stable HeLa transfectants that overexpression of the PARP DNA-binding domain increases the frequency of sister chromatid exchanges (17). Chromosomal breaks play an important role in the initiation and maintenance of the so-called unequal sister chromatid exchange/breakage-fusion-bridge cycle mechanism that is postulated to cause DNA amplification of *dihydrofolate reductase*, *CAD* (reviewed in Ref. 5), and *P-glycoprotein* (18) genes in Chinese hamster cells. On the other hand, it was shown in CO60 cells that during MNNG-induced SV40 DNA amplification, hairpin structures are formed as a primary amplification product, presumably by a so-called U-turn replication mechanism involving DNA polymerase template switching (19). According to this model, there is no requirement for chromosomal breaks; instead, arrested replication forks should promote such a mechanism. Although we could show previously that in the absence of DNA damage polyomavirus DNA replication is not limited by overexpression of the DNA-binding domain in COM3 cells (8), blocking of replication forks might occur by binding of this polypeptide to carcinogen-induced DNA strand breaks.

It has been demonstrated by several groups that genetic instability and gene amplification can result from an inappropriate entry of cells into and progression through S-phase due to the absence of p53 or Rb functions (Ref. 20 and references therein). In SV40-transformed CO60 cells, large T-antigen should bind both p53 and Rb, thus allowing carcinogen-treated cells to enter S phase. Since COM3 cells retain their competence to undergo apoptosis upon MNNG treatment,[4] we are currently investigating a possible link between increased genetic instability by *trans*-dominant inhibition of poly(ADP-ribosyl)ation and p53 activation leading to apoptosis. By using this system, we hope to contribute to the molecular understanding of the role of PARP in carcinogenesis.

Acknowledgments

We thank Prof. H. zur Hausen for his continuous support and Prof. S. Lavi for CO60 cells.

[4] J-H. Küpper, M. Müller, and A. Bürkle, unpublished observations.

References

1. Lindahl, T., Satoh, M. S., Poirier, G. G., and Klungland, A. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends. Biochem. Sci., *20:* 405–411, 1995
2. Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res., *53:* 3976–3985, 1993.
3. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. Yama/CPP32 β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell, *81:* 801–809, 1995.
4. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T-T., Yu, V. L., and Miller, D. K. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature (Lond.), *376:* 37–43, 1995.
5. Wintersberger, E. DNA amplification: new insights into its mechanism. Chromosoma, *103:* 73–81, 1994.
6. Bürkle, A., Meyer, T., Hilz, H., and zur Hausen, H. Enhancement of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced DNA amplification in a Simian virus 40-transformed Chinese hamster cell line by 3-aminobenzamide. Cancer Res., *47:* 3632–3636, 1987.
7. Bürkle, A., Heilbronn, R., and zur Hausen, H. Potentiation of carcinogen-induced methotrexate resistance and dihydrofolate reductase gene amplification by inhibitors of poly(adenosine diphosphate-ribose) polymerase. Cancer Res., *50:* 5756–5760, 1990.
8. Küpper, J. H., Müller, M., Jacobson, M. K., Tatsumi Miyajima, J., Coyle, D. L., Jacobson, E. L., and Bürkle, A. *Trans*-Dominant inhibition of poly(ADP-ribosyl)ation sensitizes cells against γ-irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine but does not limit DNA replication of a polyomavirus replicon. Mol. Cell. Biol., *15:* 3154–3163, 1995.
9. Meinkoth, J. and Wahl, G. Review. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem., *138:* 267–284, 1984.
10. Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., *132:* 6–13, 1983.
11. Lavi, S. Carcinogen-mediated amplification of viral sequences in simian virus 40-transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA, *78:* 6144–6148, 1981.
12. Ding, R., and Smulson, M. Depletion of nuclear poly(ADP-ribose) polymerase by antisense RNA expression: influences on genetic stability, chromatin organization, and carcinogen cytotoxicity. Cancer Res., *54:* 4627–4634, 1994.
13. Molinete, M., Vermeulen, W., Bürkle, A. Menissier de Murcia, J., Küpper, J. H., Hoeijmakers, J. H., and de Murcia, G. Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. EMBO J., *12:* 2109–2117, 1993.
14. Satoh, M. S., and Lindahl, T. Role of poly(ADP-ribose) formation in DNA repair. Nature (Lond.), *356:* 356–358, 1992.
15. Satoh, M. S., Poirier, G. G., and Lindahl, T. NAD(+)-dependent repair of damaged DNA by human cell extracts. J. Biol. Chem., *268:* 5480–5487, 1993.
16. Satoh, M. S., Poirier, G. G., and Lindahl, T. Dual function for poly(ADP-ribose) synthesis in response to DNA strand breakage. Biochemistry, *33:* 7099–7106, 1994.
17. Schreiber, V., Hunting, D., Trucco, C., Gowans, B., Grunwald, D., de Murcia, G., and de Murcia, J. M. A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage. Proc. Natl. Acad. Sci. USA, *92:* 4753–4757, 1995.
18. Kuo, M. T., Vyas, R. C., Jiang, L. X., and Hittelman, W. N. Chromosome breakage at a major fragile site associated with P-glycoprotein gene amplification in multidrug-resistant CHO cells. Mol. Cell. Biol., *14:* 5202–5211, 1994.
19. Cohen, S., Hassin, D., Karby, S., and Lavi, S. Hairpin structures are the primary amplification products: a novel mechanism for generation of inverted repeats during gene amplification. Mol. Cell. Biol., *14:* 7782–7791, 1994.
20. Almasan, A., Linke, S. P., Paulson, T. G., Huang, L. C., and Wahl, G. M. Genetic instability as a consequence of inappropriate entry into and progression through S-phase. Cancer Metastasis Rev., *14:* 59–73, 1995.

Trans-Dominant Inhibition of Poly(ADP-ribosyl)ation Potentiates Carcinogen-induced Gene Amplification in SV40-transformed Chinese Hamster Cells

Jan-Heiner Küpper, Marcus Müller and Alexander Bürkle

Cancer Res 1996;56:2715-2717.