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Overexpression of human poly(ADP-ribose) polymerase in transfected hamster cells leads to increased poly(ADP-ribosyl)ation and cellular sensitization to γ irradiation

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Poly(ADP-ribosyl)ation is a posttranslational modification of nuclear proteins catalyzed by poly(ADP-ribose) polymerase (PARP), an enzyme which uses NAD^+ as substrate. Binding of PARP to DNA single-strand or double-strand breaks leads to enzyme activation. Inhibition of poly(ADP-ribose) formation impairs the cellular recovery from DNA damage. Here we describe stable transfectants of the Chinese hamster cell line CO60 that constitutively overexpress human PARP (COCF clones). Immunofluorescence analysis of γ-irradiation-stimulated poly(ADP-ribose) synthesis revealed consistently larger fractions of cells positive for this polymer in the COCF clones than in control clones, which failed to express human PARP. HPLC-based quantitative determination of *in vivo* levels of poly(ADP-ribose) confirmed this result and revealed that the basal polymer levels of undamaged cells were significantly higher in the COCF clones. The COCF clones were sensitized to the cytotoxic effects of γ irradiation compared with control transfectants and parental cells. This effect could not be explained by depletion of cellular NAD^+ or ATP pools. Together with the well-known cellular sensitization by inhibition of poly(ADP-ribosyl)ation, our data lead us to hypothesize that an optimal level of cellular poly(ADP-ribose) accumulation exists for the cellular recovery from DNA damage.

Keywords: cell transfection; DNA damage; DNA repair; γ-irradiation; poly(ADP-ribose) polymerase.

An immediate response of many eucaryotic cells to DNA damage is the poly(ADP-ribosyl)ation of nuclear proteins, which is catalyzed by poly(ADP-ribose) polymerase (PARP), with NAD^+ serving as substrate (reviewed by Althaus and Richter, 1987; Lindahl et al., 1995). PARP has a DNA-binding domain comprising two zinc fingers, which mediate the binding to DNA double-strand or single-strand breaks. Binding to DNA strand breaks drastically stimulates the catalytic center of PARP, which resides in the C-terminal NAD^+-binding domain.

While poly(ADP-ribosyl)ation of histones and topoisomerases has been shown to occur in living cells, the major substrate *in vivo* is PARP itself. An increase in the concentration of poly(ADP-ribose) stimulates the activity of the enzyme poly(ADP-ribose) glycohydrolase, leading to very rapid polymer turnover in carcinogen-treated cells (Alvarez Gonzalez and Althaus, 1989). Poly(ADP-ribose) synthesis and turnover are thought to modulate cellular responses to DNA damage, i.e. DNA repair (Durkacz et al., 1980; Satoh and Lindahl, 1992; Molinete et al., 1993), DNA amplification (Bürkle et al., 1987, 1990; Hahn et al., 1990; Küpper et al., 1996), cell-cycle perturbations (Jacobson et al., 1985), malignant transformation (Borek et al., 1984; Takahashi et al., 1984; Kasid et al., 1986), necrotic cell death (Berger and Berger, 1986; Zhang et al., 1994; Heller et al., 1995) and apoptosis (Lazebnik et al., 1994; Nicholson et al., 1995; Tewari et al., 1995). Furthermore, a strong correlation between the maximal PARP activity in permeabilized mammalian lymphocytes and the lifespan of the investigated species was found (Grube and Bürkle, 1992). However, the molecular mechanisms of these effects have not been elucidated.

To understand the biological functions of poly(ADP-ribose) formation, we sought to increase the poly(ADP-ribosyl)ation capacity in cells by stably transfecting hamster cells with a human PARP-expression plasmid. Our results show that cells overexpressing human PARP can produce higher-than-normal steady-state levels of poly(ADP-ribose), and that this is associated with increased sensitivity to γ irradiation.

MATERIALS AND METHODS

Antibodies and plasmids. Rabbit anti-FII serum raised against the second zinc finger of PARP (Simonin et al., 1991) and rabbit antiserum Lima IV, recognizing the well-conserved PARP NAD^+-binding domain, were kindly provided by G. de Murcia, Strasbourg, France. Monoclonal antibody F_i23 recognizes the same epitope on the second zinc finger of human PARP as C_i9 described previously (Lamarre et al., 1988). Mouse monoclonal antibody 10H raised against poly(ADP-ribose) (Kawamitsu et al., 1984) was kindly provided by M. Miwa and T.

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Abbreviation. PARP, poly(ADP-ribose) polymerase.

Enzyme. poly(ADP-ribose) synthase (EC 2.4.2.30).

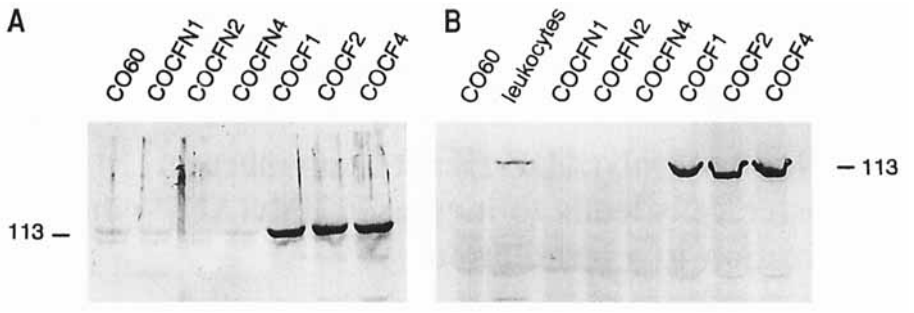

Fig. 1. Western blot analysis of PARP expression in C060 cells and transfectants derived thereof. Extracts from 10^5 cells were separated on a SDS/10% polyacrylamide gel and transferred onto Immobilone-P membranes as described in Materials and Methods. The blot was incubated with anti-FII serum (A), which recognizes endogenous hamster and exogenous human PARP, and with monoclonal antibody F_i23 (B) specific for human PARP. Mononuclear leukocytes were prepared from human blood and served as a positive control for the species-specific detection of human PARP. The blot was developed with phosphatase-conjugated immunoglobulins. The position of PARP (113 kDa) is indicated.

Sugimura, Tokyo, Japan. Phosphatase-conjugated goat anti-rabbit Ig and anti-mouse Ig were from Sigma. Cy3-conjugated donkey anti-rabbit Ig were from Dianova. Fluorescein-isothiocyanate-conjugated goat anti-mouse Ig were from Renner. Plasmid pTKneo was kindly provided by M. Boshart, Martinsried, Germany. Plasmid pPARP31 harbors the full-length human PARP cDNA (Kupper and Biirkle, 1992) under the transcriptional control of the human cytomegalovirus immediate early promoter/enhancer.

Cell culture. The simian-virus-40-transformed Chinese hamster cell line C060 (Lavi, 1981) was kindly provided by S. Lavi, Tel Aviv, Israel, and was maintained as described (Burkle et al., 1987). Stable transfectants of C060 cells were grown in medium supplemented with 800 μg/ml geneticin (GIBCO) and 10% fetal calf serum. All cell lines were grown in the absence of selection antibiotics for at least 2 days before any experiments were carried out.

Stable transfection of cells. For the generation of COCF and COCFN cell lines, exponentially growing C060 cells were transfected with 18 μg pPARP31 and 2 μg pTKneo, by means of the calcium phosphate method. Clones resistant to 800 pg/ml geneticin were analyzed by immunofluorescence and western blotting.

Immunofluorescence. To analyze poly(ADP-ribose) formation, cells grown on coverslips were subjected to *y* irradiation (^{137}Cs-Gamma Cell 1000; dose rate, 14 Gy/min; Atomic Energy of Canada Limited) in NaCl/P_i (137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogenphosphate, 8.1 mM disodium hydrogenphosphate) supplemented with 1 mM calcium chloride at room temperature. 10 min after the start of irradiation, cells were fixed with 10% ice-cold trichloroacetic acid and processed for immunofluorescence as described previously (Kupper et al., 1995). By means of immunofluorescence analysis, we had found that cellular poly(ADP-ribose) levels are peaking at this time point under our conditions. Monoclonal antibody 10H, which recognizes poly(ADP-ribose), was used as the first antibody, secondary antibodies were conjugated with fluorescein isothiocyanate.

Western blot. Crude protein extracts were prepared, separated electrophoretically and blotted as described previously (Kiipper et al., 1995). Blots were incubated overnight at 4°C with anti-FII serum diluted 1:2000 in NaCl/P_i, 0.3% Tween-20, 5% dry milk, or with hybridoma supernatant of F_i23 diluted 1:200 in NaCl/P_i, 0.3% Tween-20, 5% dry milk. Blots were developed with alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulins.

Quantitation of poly(ADP-ribose) level. This procedure was carried out as described previously (Jacobson et al., 1984). Poly(ADP-ribose) was purified from trichloroacetic-acid-insoluble cell extracts by means of boronate chromatography and was enzymatically digested to yield the nucleosides adenosine, ribosyladenosine and diribosyladenosine. Nucleosides were derivatized into fluorescent etheno compounds by chloroacetaldehyde incubation, followed by PBA-Matrex chromatography and reversed-phase HPLC (Varian) coupled with fluorometric detection (Varian). Quantitation was carried out with etheno nucleoside standards.

Quantitation of the cellular content of NAD$^+$ and ATP. Cells grown on petri dishes were *y* irradiated (14 Gy) in NaCl/P_i plus 1 mM calcium chloride at room temperature and thereafter precipitated at various times with 0.5 M perchloric acid. NAD' content was measured with a NAD' cycling assay (Jacobson and Jacobson, 1976). For determination of ATP contents, cells were lysed at different times with 8 M guanidine hydrochloride, and ATP bioluminescence assays were performed (Lundin et al., 1976).

Cell survival. Irradiation of cell monolayers and determination of cell survival was performed as described previously (Kupper et al., 1995). Exponentially growing cell monolayers were subjected to *y* irradiation at a dose rate of 14 Gy/min in NaCl/P_i at room temperature. After irradiation, NaCl/P_i was replaced with medium, and cells were incubated for 16 h under routine cell-culture conditions. Cells were trypsinized, and appropriate dilutions of cells were plated onto 10-cm petri dishes. Eight days later, colonies were fixed with 10% formaldehyde in NaCl/P_i and stained with 0.1% crystal violet. The number of colonies consisting of more than 50 cells was determined.

RESULTS

Generation of stably transfected cell lines. To obtain cell lines stably overexpressing human PARP, plasmid pPARP31, which carries the human PARP cDNA under the transcriptional control of the human cytomegalovirus immediate early promotor/enhancer, was transfected with a plasmid conferring resistance to geneticin into CO60 hamster cells. Geneticin-resistant clones were screened by immunofluorescence and western blotting. The western blot (Fig. 1 A) was developed with a polyclonal antiserum raised against the second zinc finger of human PARP (anti-FII; Simonin et al., 1991), which cross-reacts with PARP molecules from various mammalian species including hamster. In three of the clones obtained, which were designated COCF1, 2 and 4, overexpression of the 113-kDa human PARP is evident. Densitometric analysis of this western blot revealed that the level of PARP was about sevenfold that of the parental cell line

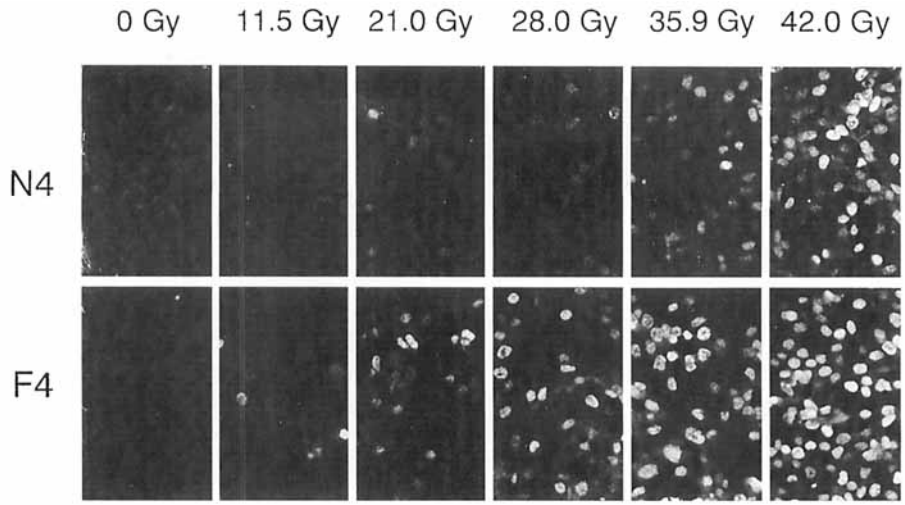

Fig. 2. Immunofluorescence analysis of clones COCFN4 and COCF4 for poly(ADP-ribose) formation after γ irradiation. COCFN4 and COCF4 cells grown on coverslips were γ irradiated in NaCl/P_i at the indicated doses. 10 min after the start of irradiation, cells were fixed and processed for immunofluoresence with mouse monoclonal antibody 10H directed against poly(ADP-ribose). The secondary antibody was labelled with fluorescein isothiocyanate.

CO60, while fivefold overexpression of human PARP in COCF clones was detected in an equivalent western blot that was probed with polyclonal rabbit antiserum LimaIV raised against the highly conserved NAD^+-binding domain of human PARP (data not shown). The latter antiserum was shown to recognize PARP of all mammalian species tested (Grube and Burkle, 1992). By means of monoclonal antibody F_i23, which recognizes human but not hamster PARP, several geneticin-resistant clones were identified that failed to express human PARP (Fig. 1 B). Three of these clones, called COCFNI, 2 and 4, served with parental C060 cells as negative controls for further experiments.

Southern blot analysis revealed different integration patterns of transfected plasmid pPARP31 in the three COCFN clones on the one hand, and in COCFl and COCF2 on the other hand, while COCF2 and COCF4 had the same integration pattern (data not shown). The latter finding was confirmed by fluorescence *in-situ* hybridization on chromosomes (data not shown). However, these two, clones which were isolated from equivalent petri dishes derived from the same transfection, behaved differently with respect to cell morphology and proliferation rate. The cell-doubling time of COCF2 was 23 h, while it is only 12 h for COCF4. COCFN4 and COCFI cannot be distinguished by Southern blot analysis, but differ with respect to human PARP expression (COCFN4 is negative, COCFI positive), cell-doubling time (11.5 h for COCFN4 and 16 h for COCFI), and with respect to their sensitivity to γ irradiation.

Analysis of poly(ADP-ribose) formation. By means of immunofluorescence analysis, overexpression of human PARP was detected in almost 100% of the COCF4 cell population, while resident hamster PARP in COCFN4 cells was not detected under these conditions (data not shown). The effect of this PARP overexpression on cellular poly(ADP-ribosyl)ation was investigated by immunofluorescence (Burkle et al., 1993) and by HPLC analysis (Jacobson et al., 1984). For immunofluorescence analysis, cells growing on coverslips were subjected to increasing doses of γ irradiation to stimulate PARP activity through the formation of DNA strand breaks. In the range of doses studied (11.5–42 Gy), poly(ADP-ribose)-specific immunostaining of cell nuclei was consistently stronger in the human-PARP-positive clones than in the negative ones. Fig. 2 shows representative

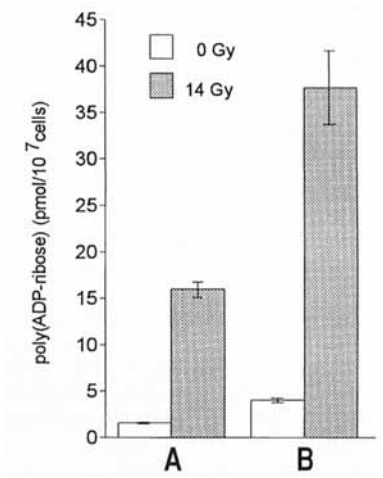

Fig. 3. Quantitative determination of poly(ADP-ribose) formation in the transfectants. Cells from clones COCFN4 (A) and COCF4 (B) were irradiated (shaded bars) or not (open bars) in NaCl/P_i. 10 min after the start of irradiation cells were precipitated by 10% trichloroacetic acid. Poly(ADP-ribose) was purified from trichloroacetic-acid-insoluble extracts by boronate chromatography and quantitated with an HPLC-based procedure (Jacobson et al., 1984). Results are from four independent samples that were assayed at the same time. Mean values and standard deviations are indicated.

experiments carried out with COCF4 cells and COCFN4 control cells, which were chosen because of their nearly identical proliferation rates (see above). In unirradiated cells of any of the clones, no nuclear poly(ADP-ribose) staining was detectable, confirming our earlier results obtained with different C060 transfectants (Kupper et al., 1995).

To quantitate the poly(ADP-ribose) levels, cells growing on petri dishes were treated with 14 Gy γ irradiation. Poly(ADP-ribose) was purified from cells by means of boronate chromatography and subjected to HPLC analysis as described in Materials and Methods. In Fig. 3 the results obtained with the clones COCF4 and COCFN4 are depicted. In clone COCF4 the basal levels of poly(ADP-ribose) are 2.6-fold higher than in the control clone COCFN4 (4 pmol/10^7 cells in COCF4 versus 1.5 pmol/10^7 cells in COCFN4). Likewise, treatment of the cells

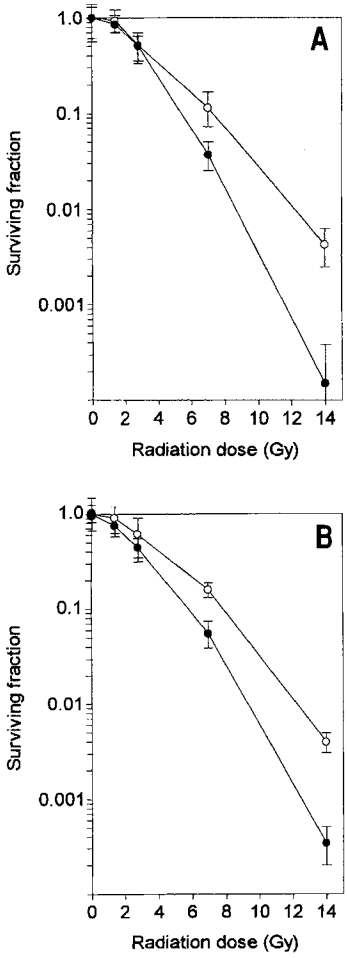

Fig. 4. Survival of COCF and COCFN clones after irradiation. (A) Exponentially growing COCF4 (filled circles) and COCFN4 cells (open circles) were γ irradiated in NaCl/P_i as indicated. After incubation for 16 h in growth medium, cells were plated in quadruplicate for the determination of cell survival. Mean values of the surviving fractions from at least three independent experiments with each clone and standard deviations are depicted. (B) Combined survival curves for COCF1, -2 and -4 (filled circles) or COCFN1, -2 and -4 cells (open circles). Surviving fractions were determined as described in (A). Mean values of the surviving fractions from at least three independent experiments done with each clone were used to calculate the combined survival curves for COCF and COCFN clones. Standard deviations are indicated.

with 14 Gy irradiation resulted in 2.3-fold higher peak levels of poly(ADP-ribose) in COCF4 cells (37.6 pmol/10^7 cells) than in COCFN4 cells (15.9 pmol/10^7 cells).

Consequences of human PARP overexpression on cell survival. Poly(ADP-ribosyl)ation has been postulated to play a role in several cellular responses to DNA damage (reviewed by Althaus and Richter, 1987; de Murcia and Menissier de Murcia, 1994; Lindahl et al., 1995). To study possible biological effects of human PARP overexpression and increased poly(ADP-ribose) formation in COCF clones, survival assays were performed after γ irradiation. In Fig. 4 A, survival data for COCF4 and COCFN4 cells are given. The human-PARP-overexpressing clone shows reduced survival after γ irradiation compared with the negative clone. Fig. 4 B shows combined survival data for all the COCF and COCFN clones. The survival curve of the parental cell line CO60 (not depicted in Fig. 4) coincides with the COCFN curve. The figure shows that radiosensitization is a general feature of

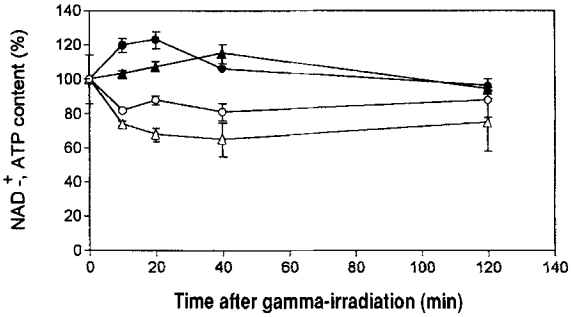

Fig. 5. ATP and NAD$^+$ contents in COCF4 and COCFN4 cells after γ irradiation. COCF4 cells (triangles) and COCFN4 cells (circles) were γ irradiated (14 Gy) in NaCl/P_i. After irradiation, cells were assayed for NAD$^+$ (open symbols) and ATP content (filled symbols) as described in Materials and Methods. For both NAD$^+$ and ATP determination, one representative experiment performed in triplicate is shown. Mean values and standard deviations are given.

the PARP-overexpressing (COCF) clones. The dose modification factor at 10% survival (LD_{10}) was 1.3.

NAD$^+$ and ATP contents after γ irradiation. According to the cell-suicide hypothesis, the poly(ADP-ribosyl)ation of cells subjected to excessive DNA damage can cause NAD$^+$, and consequently, ATP depletion and energy starvation (Berger and Berger, 1986). To analyse whether the impaired cell survival observed in the COCF clones could be due to such a mechanism, we studied cellular NAD$^+$ and ATP contents at different times after γ irradiation. For these experiments, we chose the highest irradiation dose used in the survival assays (14 Gy). Severe decreases of the cellular NAD$^+$ content were not observed in COCF4 (−35%) or in COCFN4 (−20%) (Fig. 5). Since there were no detectable decreases of ATP levels (Fig. 5), the impaired cell survival in the COCF transfectants exhibiting increased poly(ADP-ribosyl)ation cannot be explained by energy starvation.

DISCUSSION

We stably transfected a human-PARP-cDNA expression construct into the Chinese hamster cell line CO60 to see whether this could be a strategy to increase poly(ADP-ribosyl)ation in living cells above normal levels. After two subcloning procedures, we established transfectants, called COCF1, 2 and 4, that displayed constitutive overexpression of human PARP in almost 100% of the cells. Geneticin-resistant clones without detectable expression of human PARP were chosen as negative controls (COCFN1, 2 and 4). Immunofluorescence analysis of poly(ADP-ribose) formation in γ-irradiated cells revealed that the fraction of cells positive for this polymer was consistently higher in the COCF clones (Fig. 2). This was confirmed by quantitation of *in vivo* levels of poly(ADP-ribose) with an HLPC-based detection method, yielding higher poly(ADP-ribose) levels both with and without γ irradiation in COCF4 cells compared with COCFN4 control cells (Fig. 3). The result of increased poly(ADP-ribose) levels in irradiated cells is remarkable in that PARP is an abundantly expressed housekeeping protein, and the level of poly(ADP-ribosyl)ation has been thought to depend on the number of DNA breaks formed after carcinogen treatment rather than on the number of enzyme molecules available. We are currently investigating whether this increased polymer accmulation is caused by the mere overexpression of PARP or is due to qualitative differences between endogenous (hamster) and exogenous (human) PARP.

The catalytic function of PARP was attributed to a dimerized form (Mendoza Alvarez and Alvarez Gonzalez, 1993; Panzeter and Althaus, 1994). That we observed increased poly(ADP-ribosyl)ation in the COCF clones did not necessarily prove that plasmid pPARP31, which was used in the transfections, codes for a catalytically functional enzyme, since a catalytically inactive human PARP molecule heterodimerized to a hamster PARP molecule could serve as an acceptor for the polymer. However, sequence analysis of the PARP cDNA insert of pPARP31 revealed only two amino acid substitutions in comparison with one of the three sequences published (Uchida et al., 1987). First, at codon 70 we found Glu instead of Gln, the same exchange being present in PARP cDNA sequences published by two other groups (Cherney et al., 1987; Kurosaki et al., 1987). Second, at codon 762 we found Ala instead of Val, which is present in all the published sequences (Cherney et al., 1987; Kurosaki et al., 1987; Uchida et al., 1987). However, we detected Ala at this position in several independent λ NM1149 phages of the primary cDNA library of human embryonic fibroblasts that we screened (Küpper and Bürkle, 1992), and in another human PARP cDNA clone (kindly provided by G. de Murcia), which had been isolated independently and proved to be functional (Giner et al., 1992). Thus, this difference probably represents a sequence polymorphism. To definitely prove that plasmid pPARP31 encodes a catalytically active human PARP, we subcloned the cDNA insert into a yeast expression vector and transformed strain W303-1B of *S. cerevisiae*, which is considered to be devoid of poly(ADP-ribosyl)ation. In agreement with an earlier report (Kaiser et al., 1992), transformation of yeast resulted in poly(ADP-ribose) formation, which could be detected by immunofluorescence (data not shown). Moreover, we confirmed the results of Kaiser et al. showing that human PARP expression caused growth retardation in yeast, which could be reversed by adding 2.5 mM of the ADP-ribosylation inhibitor 3-methoxybenzamide to the culture medium (data not shown).

To study influences of PARP overexpression on the cellular response(s) after DNA damage, we performed survival assays on our transfectants after treatment with γ irradiation. Surprisingly, the COCF clones displayed increased radiosensitivity compared with the COCFN clones (Fig. 4). The same result was obtained when the clones were treated with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (data not shown). Since excessive PARP activation can lead to NAD^+ depletion and consequently to cell death (Berger and Berger, 1986; Heller et al., 1995; Zhang et al., 1994), we analyzed NAD^+ and ATP consumption at the highest γ dose (14 Gy) applied for the survival assays. Our results show that neither NAD^+ nor ATP depletion was the reason for the increased sensitivity in the COCF clones.

Recently, data on a similar cell system were reported. CHO-9 Chinese hamster cells and a mutagen-hypersensitive derivative thereof were stably transfected to express human PARP (Fritz et al., 1994). These transfectants displayed reduced sensitivity towards the alkylating agent methyl methanesulfonate compared with the respective parental cell line. However, since only one transfectant from each cell line was investigated, and since survival was compared with the parental cell line rather than with control clones, it is hard to exclude that the observed effects were caused by clonal variation or by the influence of selection. A strong argument that clonal variation is not the reason for the observed difference in radiosensitivity between our COCF (expressing) and COCFN (non-expressing) clones is that COCFN4 and COCF1 are of identical clonal origin, as judged from the integration pattern of the human PARP cDNA construct (data not shown), but markedly differ in their sensitivity to DNA damage. Furthermore, we recently characterized several stable transfectants of a rat cell line overexpressing human PARP cDNA from pPARP31. None of the clones that displayed increased poly(ADP-ribosyl)ation upon γ irradiation or treatment with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was protected against the inflicted DNA damage (Bernges, F., Bürkle, A., Küpper, J.-H. and Zeller, W. J., unpublished results). A report of *Schizosaccharomyces pombe* transfectants expressing human PARP showed sensitization to γ irradiation compared with untransformed fission yeast, an organism which seems not to possess a poly(ADP-ribosyl)ation pathway (Avila et al., 1994).

We and others have recently shown that *trans*-dominant inhibition of poly(ADP-ribosyl)ation is associated with sensitization to DNA damage (Küpper et al., 1995; Schreiber et al., 1995). Together with the results of this report, we hypothesize that the level of poly(ADP-ribose) accumulating upon DNA damage is strictly controlled, and any manipulation of polymer steady-state levels interferes with the cellular recovery from DNA damage. An alternative but less likely hypothesis is that there are species-specific differences in the complexity of poly(ADP-ribose). According to the protein-shuttle model proposed by Althaus, poly(ADP-ribose) has a role in the transient removal of histones from DNA to facilitate DNA repair (Althaus, 1992). It is conceivable that the polymer synthesized by human PARP is less suited to interact with hamster histones.

The study of further biochemical and biological consequences of higher-than-normal poly(ADP-ribose) levels in living cells should contribute to a better understanding of the cellular function(s) of poly(ADP-ribosyl)ation.

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