Depletion of Adenosine Diphosphate-Ribosyl Transferase Activity in Rat Liver during Exposure to N-2-Acetylaminofluorene: Effect of Thiols

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ABSTRACT

The exposure of rats to a feeding regimen containing N-2-acetylaminofluorene (2AAF) causes an accumulation of lesions on liver DNA and a progressive impairment in DNA repair capacity. We used the in vivo experimental model of Teebor and Becker (Cancer Res., 31:1–3, 1971) with the carcinogenic given to rats during four consecutive cycles, each one composed of 3 weeks of treatment and 1 week of recovery. The extent of DNA damage and repair was determined during each cycle by the alkaline elution technique. The results obtained showed that the number of alkali-labile sites in DNA is significantly enhanced after the first cycle and remains increased during following cycles.

Since ADP-riboyl transferase (ADPRT) is known to play a central role in the response to DNA damage, we investigated the effect of 2AAF on this enzyme during the carcinogenic process. The activity and the structure of ADPRT were analyzed using the activity gel and Western blot techniques. The catalytic band with a molecular weight of 116,000, clearly evident in liver extracts of control rats, was no longer detectable after one cycle of exposure to 2AAF returning progressively to an almost normal level within the last two cycles. When the aminothiol N-acetylcysteine (NAC) was added to the 2AAF diet, the extent of DNA damage was drastically reduced, and DNA repair activity preserved for a longer period. In addition, the loss of ADPRT was not observed after the first cycle, but delayed to the end of the second, indicating that NAC exerts a protective effect on DNA and on ADPRT. Such effect was not evident when NAC was substituted by glutathione.

The analysis of liver extracts on Western blot showed that the ADPRT immunoactive band was almost undetectable after the first cycle suggesting that the loss in enzyme activity could be due to a block in de novo synthesis of the enzyme and not to an inhibition of its activity.

INTRODUCTION

Malignant transformation of mammalian liver cells has been demonstrated to be a multistage process that can be initiated by the induction of DNA damage and gene mutation, and fixed by subsequent rounds of DNA and cellular replications (1–3). Experimental evidence indicates that in vivo treatment with 2AAF induces, in rat liver, the formation of DNA adducts (4, 5), the modification of several cytosolic enzyme activities and the arising of GPT positive foci (for a review see Ref. 6). This latter effect reflects the appearance and growth of “resistant” cell foci and, ultimately, of hyperplastic nodules (1–3). The prolonged exposure to the carcinogen, finally, stimulates the progression into hepatocarcinoma (7).

Although the molecular mechanisms leading to the formation of hyperplastic liver nodules are unknown, it has been demonstrated that DNA damage is induced during exposure to 2AAF and that it persists after treatment (8). This suggests that DNA excision repair capacity is altered and that this could prompt the subsequent steps of the hepatocarcinogenic process. To this end, several enzymatic activities known to be involved in DNA repair process have been analyzed: DNA-apurinic sites endonucleases (9), DNA polymerase β (10), and more recently DNA ligase (11). All these reports indicate that these enzymes were not modified in liver hyperplastic cells.

Recently it has been suggested that ADP-ribosylation of nuclear proteins is involved in DNA repair, possibly through modifications of the chromatin structure (12–14). The process is catalyzed by the enzyme ADPRT whose activity was shown to be involved in DNA repair of cells treated with DNA-damaging agents (12, 15, 16).

This work was undertaken to investigate the possible role played by ADPRT in the carcinogenic process induced in liver by 2AAF. Moreover, since we recently reported that the aminothiol NAC, a synthetic precursor of glutathione, partially prevents the DNA damage produced by 2AAF metabolites and allows the maintenance of an efficient DNA repair (17, 18), the effect of NAC on ADPRT activity and structure during the exposure to the hepatocarcinogen was studied.

After each cycle of treatment the structural properties of the enzyme were characterized by the activity gel procedure. This method allows in situ detection of active polypeptides of ADPRT after SDS-PAGE separation. The immunological features of ADPRT were studied by the Western blotting technique.

MATERIALS AND METHODS

Chemicals. N-2-Acetylaminofluorene, phenylmethylsulfonyl fluoride, and pepstatin were from Sigma. N-Acetylcysteine was a gift from Dr. V. Cicchetti (Zambon S.p.A., Milano, Italy). Glutathione was from Fluka AG. Hoechst 33258 [2-(2-(4-hydroxyphenyl)-6-benzimidazole)-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride] dye was purchased from Calbiochem-Behringer. Nitrocellulose was from Schleicher & Schuell GmbH. Newborn bovine serum was purchased from Flow; alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L), alkaline phosphatase color development reagents NBT and BCIP were obtained from Bio-Rad. A rabbit antisera raised against highly purified calf thymus ADPRT was kindly donated by Drs. M. I. Ittel and C. Niedergang, CNRS Strasbourg, France. DE 81 paper was obtained from Whatman. Prestained protein molecular weight standards were from BRL. Adenine-2,8-[3H]NAD (25 Ci/mmol) and adenine-2,8-[32P]NAD (1000 Ci/mmol) were from New England Nuclear; adenosine-8-[32P] (50 Ci/mmol) and [3H]TTP (50 Ci/mmol) were from Amersham. Calf thymus DNA was activated to about 3–5% solubility by DNase I.

Male Wistar rats, weighing 80–100 g, were purchased from Morini

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ADPRT DEPLETION IN LIVER OF RATS EXPOSED TO 2AAF

RESULTS

Effect of 2AAF Treatment on the Elution Kinetics of Rat Liver DNA. The four-cycle experimental model followed for the discontinuous feeding regimen used for rat treatment was essentially that devised by Teebor & Becker (19) and described in “Materials and Methods.” The 2AAF-containing diet was supplied during the first 3 weeks of each cycle and discontinued during the fourth week to allow recovery. Liver samples to be analyzed were obtained from rats sacrificed at the end of the third and of the fourth week of each cycle.

In order to assess the extent of DNA damage induced in rat liver the alkaline elution kinetics of DNA was determined for each liver sample as described in “Materials and Methods.” The results obtained for each of the four cycles are shown in Fig. 1, A-D, respectively. The profiles are obtained by plotting the DNA fraction retained on membrane filter against the volume of eluted buffer. The slope of the curve is directly proportional to the number of DNA alkali-labile sites. The elution curves corresponding to the third week of treatment of each cycle represent the total amount of DNA damage produced by the carcinogen whereas those related to the fourth week of the cycle indicate the extent of residual DNA damage. Thus the difference between these two sets of data corresponds to DNA repair activity occurring during the recovery period. It is evident that the treatment with 2AAF induces a striking decrease of DNA fraction retained on filter which is already evident after the third week of the first cycle. The extent of DNA damage is notably reduced when NAC is concomitantly supplemented with the carcinogen. However this protective effect is no more evident during the last two cycles. The data indicate that the extent of DNA damage not repaired during the recovery period

![Fig. 1. Alkaline elution kinetics of liver DNA from rats exposed to a discontinuous feeding regimen with 2AAF. The elution profiles are reported in each panel as a function of the treatment cycle. A-D, obtained for each of the four cycles, respectively, and referring to: DNA from control rats ( ); DNA from 2AAF-treated animals sacrificed on the 3rd (A) and on the 4th week (A); DNA from 2AAF-treated rats supplemented with 0.1% NAC, sacrificed on the 3rd (C) and on the 4th week (B) of each cycle. The elution curves are obtained from the semilog plot of DNA fraction retained on the membrane filter (log scale) versus the eluted volume of alkaline buffer. Values, mean of at least five triplicate experiments.](image)
ADPRT Depletion in Liver of Rats Exposed to 2AAF tends to increase with the length of the treatment.

These observations were confirmed by calculating for each curve the elution rate constant, as reported in Fig. 2. The total amount of DNA damage, measured at the end of the third week of each cycle, is already significantly increased over controls after the first cycle \( (p < 0.001) \) and tends to remain at the same level during subsequent exposures. On the first and second cycle only 50% of DNA damage is repaired in the recovery period and the addition of NAC to the 2AAF diet exerts a protective effect by further reducing the extent of damage and restoring it to values close to those found for controls.

During the last two cycles, it appears that the extent of DNA damage repaired in the recovery period is significantly reduced and the protective effect of NAC drastically affected.

Effect of 2AAF Treatment on Rat Liver ADP-Ribosyl Transferase. The activity and molecular weight of ADPRT were analyzed in liver extracts obtained after the recovery period of each of the four cycles by means of the activity gel procedure and of the Western blotting technique. The activity gel analysis of ADPRT of control and 2AAF-treated rats is shown in Fig. 3A. A catalytic band with a molecular weight of 116,000 was well evident in control extracts (lane 1) whereas after the first cycle the active band was no longer detectable (lane 2), reappearing progressively only in following cycles (lanes 3–5).

In a second series of experiments rats were fed with 2AAF and 0.1% NAC or 0.1% GSH (Fig. 3, B and C). When NAC was supplemented to the diet the active band of ADPRT was still evident after the first cycle (Fig. 3B, lane 2) becoming barely detectable after the second cycle (lane 3) and returning to normal levels after the third and fourth cycle (lanes 4 and 5). When NAC was substituted by GSH the protective effect was not evident and the activity gel pattern obtained (Fig. 3C) was very similar to that observed for treatment with 2AAF alone (compare A and C of Fig. 3).

In order to understand whether the loss in the enzymatic activity observed in 2AAF-treated rats was due to an inhibition of ADPRT or to a block in the synthesis of the enzyme, the same extracts were analyzed by the Western blot technique using a polyclonal antibody against ADPRT. The results of a typical experiment are shown in Fig. 4. An immunoreactive peptide with a molecular weight of 116,000 was clearly recognized in untreated rat liver extracts (lane 1) whereas it became almost undetectable after the first and the second cycle of 2AAF treatment (lanes 2 and 3). This indicates that the loss in the catalytic band observed in activity gels corresponds to a lack of ADPRT protein. During the two following cycles, when the activity band of ADPRT reappeared, also the Mr 116,000 immunoreactive peptide was evident (lanes 4 and 5).

The Western blot analysis was even carried out on extracts from liver of rats treated with 2AAF and NAC (Fig. 4). Also in this case a close correlation between the presence of immunoreactive peptides and activity bands was obtained confirming the protective effect of the aminothiol during the first cycle.

Analysis of Adenosine Deaminase and DNA Polymerases in Control and Treated Rat Liver. We have in addition determined the level of adenosine deaminase and of DNA polymerases \( \alpha \) and \( \beta \). The specific activity of adenosine deaminase ranged from 4.2 to 6.6 U/mg protein in controls and from 4.5 to 7.6 in livers of treated animals. The values of DNA polymerase \( \alpha \) ranged within 0.06–0.09 U/mg protein in controls and treated livers and those for DNA polymerase \( \beta \) were computed between 0.13 and 0.20 U/mg protein in the same samples. These results suggest that other essential cellular proteins are not signifi-
Carcinoma(s) (7). Many lines of evidence, obtained by this relationship between DNA repair capacity and ADPRT activity so far observed. Model, support the aforementioned view (9-11, 27) although positive foci, liver hyperplastic nodules and ultimately hepato-cytes are repaired during the recovery period of the first two cycles and that repair capacity is significantly affected during the last two cycles.

When N-acetylcysteine, a free radical scavenger aminothiol, well known as a precursor of glutathione and as a cysteine pool supplier, was supplemented to the diet with 2AAF, the extent of DNA damage in the first two cycles was notably reduced and led to an almost complete DNA repair, although the protective effect was not lasting in later cycles.

The activity and the structure of ADPRT was analyzed using activity gel and Western blot procedures. The catalytic band of Mr 116,000, clearly evident using liver extracts of control rats, was no longer detectable after one cycle of 2AAF exposure. When NAC was added to the 2AAF diet, the loss of the ADPRT activity band was not observed after the first cycle but only to the end of the second. No significant protective effect was exerted when NAC was substituted by GSH.

The analysis of the same extracts on Western blot showed that the immunoreactive peptide of Mr 116,000 was clearly recognized in untreated rat liver whereas it was undetectable after the first and second 2AAF treatment cycles, indicating that the loss of the catalytic band observed in activity gels was due to a lack of ADPRT protein and not to an inhibition of the enzyme.

These results would suggest that in this system ADPRT is not strictly related to DNA repair capacity since its activity tends to drop when cellular response to DNA damage is apparently still effective. However it should be noted that after one cycle of treatment about 50% of DNA lesions are not repairable, suggesting that the repair process is already impaired. In addition, it has been demonstrated that ADPRT response to DNA damage occurs shortly after cellular exposure to genotoxic agents (12, 15). Our observations, on the contrary, refer to a late effect possibly related to specific regulatory processes involved in the initiation of the hepatocarcinogenesis (29, 30).

An additional interesting result of this study is the observation that ADPRT activity, after an apparent complete loss in early cycles, reappears progressively during the ongoing treatment. This seems to be related more to an increasing number of “resistant” cells than to a reversal of the inhibitory effect in normal hepatocytes (29). In fact it has been shown that altered hepatocytes in hyperplastic liver nodules, such as those induced by continuous or intermittent exposure of rats to 2AAF, present characteristic cellular and physiological patterns and are phenotypically different from normal liver cells at any stage of their development (29, 30). An increased excretion of 2AAF metabolites from liver with many altered cells could be responsible for a decreased binding of 2AAF to DNA, RNA, and proteins in resistant cells of liver nodules (29, 31).

Moreover, it must be noted that these transformed cells are able to grow in an environment that severely hinders the replication of the bulk of the normal hepatocytes (32). Therefore it can be argued that the 2AAF-induced loss in ADPRT could be due to a specific inhibition of ADPRT de novo synthesis in

**DISCUSSION**

Considerable evidence indicates that the accumulation of alterations in DNA may be the basic event in the initiation of carcinogenesis (26, 27). Among the mechanisms responsible for this phenomenon of particular importance is a decreased capacity in DNA repair activity.

The relationship between DNA repair and carcinogenesis was studied in several laboratories using the in vivo model of Teebor and Becker (19). This experimental system is based on a discontinuous feeding regimen with the carcinogen 2AAF given to rats during four consecutive cycles each one composed of a 3-week treatment and 1-week recovery. A complete four-cycle exposure is known to induce the development of GGT-positive foci, liver hyperplastic nodules and ultimately hepatocarcinoma(s) (7). Many lines of evidence, obtained by this model, support the aforementioned view (9→11, 27) although no alterations in the different steps of DNA repair have been so far observed.

Since it has been demonstrated that ADP-ribosyl transferase plays a central role in the modulation of cellular response(s) to DNA damage(s) (12, 13, 28), we investigated in rat liver the relationship between DNA repair capacity and ADPRT activity during the exposure to 2AAF. In addition, the extent of DNA damage and ADPRT activity were followed early during exposure to the carcinogen and also at later stages that reflect the arising and persistence of putative premalignant cell foci and nodules. The total damage to liver DNA was evaluated using the alkaline elution procedure and calculating the elution rate constants after the treatment and the recovery periods of each cycle.

The difference between these two sets of data corresponds to the DNA repair activity which occurred during the recovery period. The results obtained clearly show that the number of alkali-labile sites in DNA is significantly increased after the first cycle of treatment and tends to remain enhanced during following cycles. It also appears that only 50% of DNA lesions are repaired during the recovery period of the first two cycles and that repair capacity is significantly affected during the last two cycles.

**Fig. 4. Western blot analysis of ADPRT in rat liver extracts.** The animals were exposed to a four-cycle discontinuous feeding regimen containing 0.05% 2AAF (A) or 0.05% 2AAF + 0.01% NAC (B). Lanes: 1, control extracts; 2–5, extracts obtained after the 1st, 2nd, 3rd, and 4th cycle of treatment, respectively.

Significantly affected by the treatment and that the effect obtained for ADPRT is a specific one.
normal hepatocytes during the period of time in which the number of “resistant” cells is not yet considerably increased. The progressive reappearance and enhancement in ADPRT level during successive cycles is concomitant to the increase in GGT-positive cells (18) thus suggesting that the return of the enzymatic activity is solely due to the remarkable burst of 2AAF-resistant cells.

A direct modification of the enzyme by 2AAF metabolites appears as unlikely since our results demonstrate that the molecular mass of ADPRT remained unchanged and that the activity of other enzymes such as adenosine deaminase and DNA polymerases α and β was unaffected.

Finally, it is known that 2AAF stimulates DNA repair synthesis possibly resulting in an increased accessibility of the genome. Thus a specific modification in genomic DNA sequences should be considered since it was evidenced that active genes are more susceptible to be modified by chemical agents (33). To further understand whether the expression of this important enzyme is blocked at the level of the synthesis of its mRNA or by directly damaging its gene, it is instrumental to make use of specific DNA probes resulting from the cloning of the cDNA, which has been very recently accomplished (34, 35).

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REFERENCES

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