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Constitutive Expression and Inducibility of Plasminogen Activator in Mer$^-$ Glioblastoma A1235 Cell Line and in Mer$^+$ A8 Cells Transfected with Bacterial *ada* Gene

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Summary

The alkylation repair deficient (Mer$^-$ phenotype) cells produce higher levels of proteolytic enzyme plasminogen activator (PA) after treatment with alkylation agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The purpose of the present study was to investigate the induction and the stabilization of urokinase plasminogen activator *(upa)* transcripts in Mer$^-$ A1235 and in Mer$^+$ A8 cells which harbor bacterial *ada* gene, following MNNG treatment. Northern blotting experiments revealed an increased amount of *upa* transcripts in Mer$^-$ A1235 human glioblastoma cell line treated with 5 μM MNNG. However, no induction of *upa* transcript was detected in its Mer$^+$ counterpart cells (A8 cells) following the same drug treatment. Studies with inhibitors of RNA and protein synthesis (actinomycin D and cycloheximide, respectively) indicate that the induced increase in amount of *upa* mRNA was due to enhanced transcription of the *upa* gene. Furthermore, they revealed that the turnover of the *upa* mRNA is relatively low (half-life > 6 h). These results demonstrate increase in transcription of *upa* gene in human glioblastoma Mer$^-$ cells after MNNG treatment. They suggest that induction of the *upa* gene might therefore be included in a cellular response to DNA damage.

Key words: A1235 human glioblastoma, Mer phenotype, MNNG, induction, urokinase plasminogen activator

Introduction

Induction of various genes by a wide range of agents that cause cytotoxic stress has been reported in mammalian cells (*1-3*). New gene transcripts following various DNA damage include protooncogenes, DNA binding transcription factors, proteases and a variety of other gene products (*4, 5*).

Urokinase plasminogen activator (uPA) is a specific serine protease closely associated with cellular transformation, neoplasia, tumor promotion and metastasis (*6-8*). It has been reported that alkylating agent (MNNG) induces production of uPA in Mer$^-$ cells, but not in Mer$^+$ cells (*9, 10*). This observation suggests that uPA induction in alkylation-repair deficient cells is related to unrepaired DNA damage, but a mechanism and a role of uPA in the DNA repair has not been clarified by now.

We have previously shown that human glioblastoma A1235 transfectants expressing the *E. coli ada* gene (A8 cells) restored the resistance to MNNG alkylating

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agents due to the bacterial methyltransferase activity. In addition, those transfectants showed significantly lower extracellular uPA activity following MNNG treatment as compared to Mer$^-$ A1235 parental cells (*11*).

The purpose of the present study was to examine the induction and the stabilization of *upa* transcripts in this cell system (Mer$^-$ A1235 and Mer$^+$ A8 cells which harbor bacterial *ada* gene) following MNNG treatment. Here, we show that increased uPA activity in A1235 cells observed previously (*11*) is due to increased transcription of the *upa* gene after alkylation. In addition, only constitutive level of *upa* transcript has been observed after the same MNNG treatment in A8 cells.

It has been shown that N-nitrosourea based prodrugs were designed in such a way that their activation depended on tumor-associated proteases. Moreover, the data obtained confirmed that tumor cells expressing PA became significantly more sensitive to such prodrugs in the presence of plasminogen (*12*). As we found in this paper, if PA induction is inversely associated with the capacity of alkylation repair, and if it appears to be a general principle, it could be a very good additional experimental system for selection of antineoplastic drugs in future.

Material and Methods

Cell lines and cell treatment

The Mer$^-$ A1235 human glioblastoma cell line (a gift from S.A. Aaronson, NIH, Bethesda, MD) is incapable of repairing O^6-methylguanine product in its DNA due to the absence of detectable O^6-methylguanine-DNA methyltransferase (MGMT) activity. This cell line is sensitive to killing by MNNG (Mer$^-$ cells) (*13*). A8 cell line is a stable transfectant of A1235 cell line expressing bacterial *ada* gene. Transfection and selection of transformants has been described previously (*11*). According to its ability to survive toxic effects of MNNG, A8 cell line is considered to be Mer$^+$. Cells were cultured in Dulbeco's modified Eagle's medium supplemented with 10 % fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 units/mL) and L-glutamine (0.03 %) and incubated in humidified 5 % CO_2 at 37 °C. A8 cells were grown in presence of 200 μg of the aminoglycoside G418 per mL.

Cells were seeded 24 hours before the treatment to appropriate cell density and were treated with 5 μM of MNNG, 1 μg/mL of actinomycin D or 2 μg/mL cycloheximide, or with a combination of MNNG and actinomycin D. MNNG stock solutions (1 mg/mL) in absolute ethanol were stored at –20 °C for up to 1 year without any loss of potency; dilutions were made in DMEM without serum. Since MNNG has a reported half-life of about 15 min in tissue culture medium (*14*), the cells were not washed after MNNG addition.

RNA isolation and Northern blot analysis

At indicated time points, total cellular RNA was extracted according to the method of Chomczynski and Sacchi (*15*) and 20 μg of each sample were electrophoresed on a formaldehide-agarose gel, following capillar transfer to Hybond N$^+$ membrane (Amersham). Blots were hybridised with human *upa* and β-*actin* cDNA probes. Probes *upa* (1400 bp) and β-*actin* (618 bp) were amplified from total cDNA mixture by RT-PCR reaction using following primers sets:

- for β-*actin*
  5´-ATCATGTTTGAGACCTTCAACACC-3´ and
  5´-CATCTCTTGCTCGAAGTCCAGGGC-3´,
- and for *upa*
  5´-CCAAGCTTGCCACCATGAGAG-3´
  and 5´-GGGG-ATCCTCAGAGGGCCAGG-3´.

Probes were ^{32}P-labeled by Promega labeling kit according to manufacturers instructions. Prehybridisation was preformed for three hours at 42 °C in prehybridisation solution containing 200 μg/mL salmon sperm DNA. The content of prehybridization solution was: 50 % formamide, 1 M NaCl, 1 % sodium dodecyl sulfate (SDS) and 10 % dextran sulphate. The same solution was used for hybridisation with addition of ^{32}P-labeled probes. Following overnight hybridisation, membranes were washed twice in 2 × SSC (0.3 M sodium cloride, 0.03 M sodium citrate), 0.5 % SDS, once with 1 × SSC, 0.25 % SDS and once with 0.1 × SSC (all at 64 °C), and subsequently exposed to X ray film (Amersham). Signal intensities were determined by densitometer. Probes were stripped for rehybridisation by washing of membranes in boiling 1 % SDS, 0.1 × SSC solution for 5 minutes.

Results and Discussion

Identification of upa as an MNNG-inducible gene

We have previously shown that alkylating agent MNNG induces the uPA enzymatic activity in Mer$^-$ A1235 cells, while no induction was observed in Mer$^+$ A8 cells (*11*). In order to test whether the enhanced production of uPA in A1235 cells was due to an increased level of mRNA, the expression of the *upa* gene was studied by Northern blot analysis.

Northern blot analyses using ^{32}P-labeled probes from a previously described authentic human *upa* cDNA isolate (*16*) confirmed induction of transcription of the *upa* gene (molecular weight approximately 2.5-kilobases) following exposure of human A1235 glioblastoma cells to 5 μM MNNG (Fig.1). Differences in mRNA transcripts expression were not due to variations in the amounts of RNA loaded since similar signals of the human *β actin* mRNAs were observed in RNA from untreated as compared to MNNG-treated A1235 cells (Fig. 1).

Increase of uPA activity was observed in many cells exposed to alkylating agents. The mechanism of induction was hitherto unknown. The observed increase of uPA activity could be due to increased transcription or posttranscriptional and posttranslational alterations. Here, we show that the *upa* gene is inducible by alkylating treatment. Accumulation of *upa* mRNA was observed in Mer$^-$ A1235, but not in Mer$^+$ A8 cells. Our data strongly suggest that increased uPA activity observed previously (*11*) is due to increased transcription of *upa* gene.

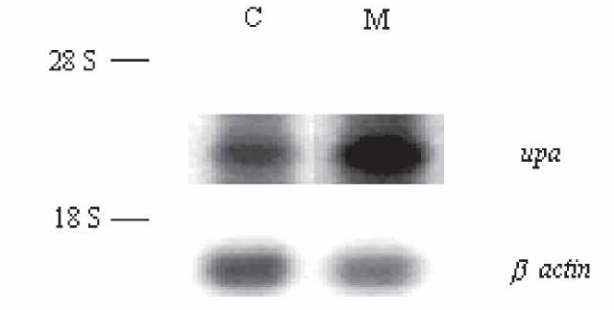

Fig. 1. Induction of *upa* mRNA by MNNG; total RNA (20 μg) isolated either from untreated A1235 cells (C, control) or 48 h after MNNG treatement (M) was fractionated on a 1 % denaturing agarose gel and transferred onto a nylon-based filter (Amersham). Samples were then hybridized with ^{32}P-labeled *upa* and *β actin* probes. Hybridized blots were then washed and prepared for autoradiography as described in »Materials and Methods«

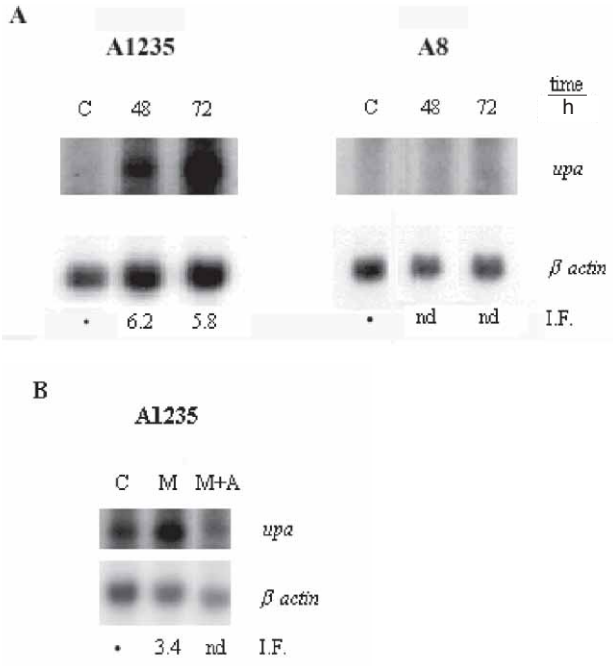

Fig. 2. **(A)** Northern blot analysis of *upa* mRNA of A1235 and A8 cells treated with MNNG; RNA was extracted in indicated time intervals after addition of 5 μM MNNG to the medium. The membrane was reprobed with *β actin*. The induction factor (I.F.) represents relative amount of *upa* mRNA (*upa/β actin*) after treatment, in relation to the corresponding untreated control (C) that was included in each experiment
(B) Inhibition of MNNG-induced *upa* transcription by actinomycin D (1 μg/mL); cells were treated with 5 μM MNNG and incubated for 24 h (M). Actinomycin D was added immediately after MNNG addition (M+A). n.d. not detectable induction

Further, results show that the *upa* mRNA level in A1235 cells increases after the cell treatment (Fig 2A). To the contrary, there was no detectable induction of *upa* mRNA transcripts in A8 cells, which express bacterial *ada* gene. The relative abundance of the *upa* mRNA in treated cells was determined by densitometry measurement and compared by measuring the *β actin* mRNAs.

In addition, *upa* mRNA accumulation in A1235 cells was inhibited completely by actinomycin D given immediately after addition of MNNG to the medium (Fig. 2B). This strongly indicates that increased *upa* mRNA levels were due to stimulation of transcription of the *upa* gene after alkylation. Accumulation of the *upa* transcripts in A1235 cells is a late response to DNA damage.

Stability of upa mRNA in A1235 and A8 cells

In order to analyze the turnover of *upa* mRNA, we assayed its amount in A1235 and A8 cells treated with actinomycin D (to block RNA synthesis) or with cycloheximide (to block protein synthesis). For controls, filters were rehybridized with *β actin*. *β actin* mRNA is relatively stable, with half-life of 8 h (*17*). As shown in Fig. 3, in A1235 and A8 cells amount of *upa* mRNA did not become significantly reduced during 6 h incubation period with actinomycin D (compared with *β actin*). This indicates that the *upa* mRNA is quite stable, with a half-life longer than 6 h. Treatment of the A1235 cells with MNNG prior to actinomycin D gave essentially the same results as actinomycin D treatment alone, suggesting that alkylation does not significantly reduce *upa* mRNA stability.

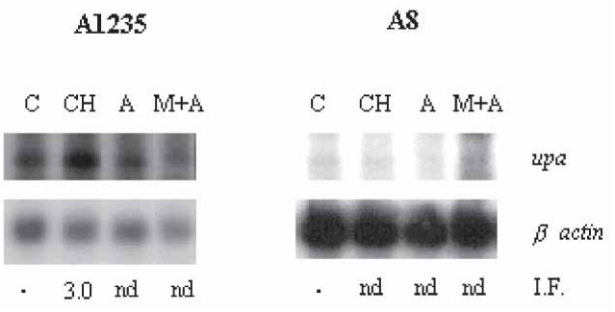

Fig. 3. Blot hybridization of RNA of A1235 and A8 cells incubated for 6 h in the presence of actinomycin D (1 μg/mL) (A) or cycloheximide (2 μg/mL) (CH); actinomycin D was added immediately after MNNG addition (M+A). After hybridization with *upa* cDNA, the filters were rehybridized with ^{32}P-labeled *β actin*. C, untreated control
n.d., not detectable induction

The stability of *upa* mRNA differs from several DNA damage-inducible short-lived transcripts, e.g. c-*fos* and c-*jun*, whose transcription is maximal between 2 and 8 h after the cell treatment (*18,19*). Another difference from these DNA damage-inducible genes (*19-22*) is that transcriptional activation is maintained for long period of time (up to 72 h after alkylation) which partly can be due to the high stability of the transcript. This different kinetics of induction suggests the existence of different regulatory pathways controlling gene expression in response to DNA damage in mammalian cells.

For several mRNAs, including c-*myc* and c-*fos* mRNA, cycloheximide was shown to give rise to mRNA accumulation due to prevention of mRNA degradation (*18,23*). In our experiments with A1235 cells, cycloheximide (treatment of cells for 6 h) induced a marked accumulation of *upa* mRNA (Fig. 3A). To the contrary, in

the experiments with A8 cells, cycloheximide did not alter the amount of *upa* transcripts (Fig. 3B). This suggests that the *upa* gene is transcribed in A1235 cells at a higher constitutive level than in A8 cells, but we are not able to discuss this discrepancy at present.

In conclusion, our present studies demonstrate significant up regulation of urokinase plasminogen activator in human glioblastoma Mer$^-$ cells after MNNG treatment. They suggest that induction of the *upa* gene might therefore be involved in a cellular response to DNA damage. The mechanism of this up regulation is the subject of our further research.

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Konstitutivna ekspresija i inducibilnost plazminogen aktivatora u Mer$^-$ A1235 stanicama glioblastoma te u Mer$^+$ A8 stanicama transfektiranih s bakterijskim *ada* genom

Sažetak

Stanice manjkave u popravku alkilacijskog oštećenja (Mer-fenotip), nakon obradbe alkilirajućim agensom N-metil-N′-nitro-N-nitrozogvanidinom (MNNG) proizvode povećanu količinu proteolitičkog enzima plazminogen aktivatora (PA). Svrha ovoga istraživanja bila je ispitati indukciju i stabilizaciju transkripata urokinaznog plazminogen aktivatora (*upa*) nakon obradbe s MNNG, u Mer$^-$ A1235 i u Mer$^+$ A8 stanicama koje imaju bakterijski *ada* gen. Hibridizacijom RNA (»nothern blotting«) nađena je povećana količina *upa* transkripata u Mer$^-$ A1235 staničnoj liniji ljudskoga glioblastoma obrađenoj s 5 mM MNNG. Međutim, u Mer$^+$ stanicama (A8 stanice), nakon iste obradbe nije detektirana indukcija *upa* transkripata. Istraživanja s inhibitorima sinteze RNA i proteina (aktinomicin D i cikloheksimid) pokazuju da je inducirani porast *upa* mRNA uzrokovan povećanom transkripcijom *upa* gena. Nadalje, ona pokazuje da je izmjena *upa* mRNA vrlo mala (vrijeme poluraspada > 6 sati). Ovi rezultati pokazuju povećanje transkripcije *upa* gena u ljudskim Mer$^-$ stanicama glioblastoma nakon obradbe s MNNG. Oni upućuju na to da bi indukcija *upa* gena mogla, zbog toga, biti uključena u stanični odgovor na oštećenje DNA.