Identification of Mutagenic Site of c-H-ras Oncogene Damaged by N-acetoxyaceteylaminofluorene (AAAF)*

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A molecularly cloned human cellular H-ras (c-H-ras) oncogene (pcb N1 plasmid) was treated with N-acetoxyaceteylaminofluorene (AAAF) in vitro and subcloned into E.coli. This was done to identify the mutational changes at specific codons of the gene. Cytidine nucleotides were identified as the major AAF binding site of the DNA adduct formed. Base changes in codons 12 and 61 were determined by the analysis of restriction fragment length polymorphism (RFLP) and site specific oligonucleotide hybridization. RFLP was observed due to the loss of the HpaI recognition site at codon 11 and 12 of AAF-treated c-H-ras gene. Hybridization of AAF treated c-H-ras with 32P-labeled oligonucleotide probes for the mutant alleles of codon 61 showed no substitutions at codon 61. From these results, it is assumed that AAF treatment in vitro caused mutation at codon 12 but not at codon 61 of the c-H-ras oncogene and that codon 12 is the primary target of mutation by AAF.

Key Words: c-H-ras oncogene, AAAF, mutation

Most chemical carcinogens are known to exert their biological activity through metabolic activation to ultimate carcinogens that can interact with cellular macromolecules including DNA (Miller, 1978). It is now widely recognized that the interaction between the ultimate carcinogens and cellular DNA is a crucial event in the process of neoplastic transformation (Irving, 1973; Lefevere et al. 1978).

The damage of DNA by chemical carcinogens may result in point mutation, deletion, translocation or gene amplification (Weinstein, 1981), but the type of damage is varied with the different kinds of carcinogens and DNAs.

Cellular H-ras (c-H-ras) oncogene activation was observed in various human tumors (Pulciani et al. 1982; Sekuga et al. 1983; Brown et al. 1986; Sinha et al. 1988; Beer and Pitt, 1989), and mutations in the activated c-H-ras have been observed in various tumors induced by chemical carcinogens (Hall et al. 1983; Balmain, 1984; Cohen and Levinson, 1988; Guerro and Pellicer, 1987, Marien et al. 1989). The activation of the cellular ras genes was thought to be due to the point mutations at codons 12 or 61 (Skumar et al. 1983; Zarbl et al. 1985).

N-acetoxyaceteylaminofluorene (AAAF), a metabolite of 2-acetamidofluorene (AAF) has been known as an ultimate hepatocarcinogen that can directly interact with DNA (Miller, 1970; Harvan et al. 1977), and it is known that AAAF causes activational mutations of c-H-ras in animals (Vosden et al. 1986; Wiseman et al. 1986) although the mechanisms of activation remains unclear.

In the present study, AAF was treated with the plasmid harboring c-H-ras oncogene in vitro and the structural alteration of the gene was analyzed by restriction fragment length polymorphism (RFLP) and selective oligonucleotide hybridization.

MATERIALS AND METHODS

Modification of the Plasmid

Plasmids pcb N1 (ATCC, USA), which contains 6.4kb of c-H-ras gene originating from human fetal
liver cells were treated with 10μM of AAAF. This plasmid was then digested with BamHI restriction enzyme and after agarose gel (1.0%) electrophoresis the c-H-ras gene fragment was isolated by extraction with Gene Clean II (Boehringer Mannheim, Germany). The damaged c-H-ras gene fragment was ligated into pBR322 at BamHI site and subcloned into E.coli (DH1).

Detection of AAAF-DNA Adduct

AAAF treated plasmid DNA was extracted 3 times with an equal volume of ether and the DNA was precipitated with 2 volumes of ethanol. N-acetyl-N-(deoxyguanosine-8-yl)-2-aminofluorene (dGp-C₈ (N₂-AAF)) was prepared by the treatment of dGMP (15mM) with AAAF (10mM) in 200μl of 50mM sodium acetate (pH 4.8) in 50% ethanol. This reaction was kept at 38°C for 1 hour. This was followed by ether extraction according to the method of Gupta et al. (1982). Control or carcinogen modified DNAs were degraded into dNMP by inoculating 1μg of DNA with 0.2 units of each DNAase I and spleen exonuclease (Sigma Chem. Co., USA) dissolved in 10μl of 20mM sodium succinate with 10mM CaCl₂, pH 6.0. The reaction was kept at 38°C for 2 hours.

DNA digests and dGp-C₈ (N₂-AAF) were labeled with ³²P by incubating a 2.0μl aliquot of the freshly prepared digest (~0.5nmol dNMP) with 1μl of 0.1M MgCl₂, 0.1M dithiothreitol, 10mM spermidine, pH 9.0, 4μl of [γ-³²P] ATP(150-200μCi), 1.2μl of 5mM cold ATP and 1.1μl of T4 polynucleotide kinase (3.0U/μl). The reaction mixture was incubated at 38°C for 30min. The ³²P-labeled dNMP and AAAF modified dNMP were mapped by silica gel thin layer chromatography according to the method of Kriek et al. (1967), using benzene: methanol (9:1) for the first dimensional solvent system. For autoradiography, Fuji X-ray film was placed in contact with polyethylene wrap film covered on a thin layer plate and exposed for 2 hours at -70°C.

Subcloning of AAAF Treated c-H-ras Gene

AAAF-treated plasmids were digested with BamHI and were subjected to agarose gel electrophoresis. The 6.4kb fragments corresponding to c-H-ras gene were isolated and inserted into the BamHI site of pBR322 by the method of Davis et al. (1986), and the plasmids were used for the transformation of E.coli (DH1). Transformants containing the c-H-ras sequence were selected by the analysis of the restriction pattern after the digestion with BamHI and Sau3A restriction enzymes.

Detection of RFLP

In order to identify the mutational changes at codon No. 12 of c-H-ras, plasmids isolated from the transformants were digested with SacI. The 3kb fragments were recovered from the agarose gel after electrophoresis. The DNA fragments (3kb) were digested with XbaI restriction enzymes followed by agarose gel (1.5%) electrophoresis. The DNA fragments of 850 bp containing the first exon of c-H-ras were isolated from the gel using Gene Clean II.

The 850bp DNA fragments were further digested with HpaII in order to identify the length polymorphism due to the change at codon 11 and 12 which contains the CCGG sequence of the wild type of c-H-ras, a recognition site of HpaII.

Oligonucleotide Hybridization

Oligonucleotide probes, complementary to the codon 61 of normal or mutant alleles of the human c-H-ras (oncogene battery) were end labeled with ³²P using [γ-³²P]-ATP according to the method of Bos et al. (1984). The AAAF-treated plasmids were digested with SacI and subjected to agarose gel electrophoresis. The DNA fragments in the gel were blotted on nitrocellulose paper (Sigma Chem. Co., USA) according to the method of Southern (1975). The membrane was then baked for 2 hours at 80°C followed by prehybridization and hybridization with ³²P-labeled probes each performed for 24 hours at 51°C as described by Lathe (1985). The membrane filter was washed with 250ml of 2 x SSC buffer (0.3M NaCl, 0.03M sodium citrate, pH7.0) prewarmed to incubation temperature, for 5min, then washed twice with 250ml of fresh buffer at 10°C lower than the incubation temperature.

The washed membrane was blotted between two pieces of Whatman 3MM filter paper and air dried. Autoradiography was performed at -70°C with Fuji X-ray film.

RESULTS

Identification of AAAF-DNA Adduct

The purity of AAAF was confirmed by silica gel thin layer chromatography using a solvent system of benzene: methanol (9:1. V/V). AAAF had a Rf value of 0.45 (Fig. 1) that is consistent with the value indicated by the supplier (Chemsys Science Laboratories, Lenexa, Kansas, USA). The major AAAF-DNA
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Adduct was identified as dGp-C (N-AAF) with other minor AAAF derivatives (Fig. 2).

**Restriction Analysis of AAAF Treated c-H-ras Gene**

The restriction map for the normal pbc N1 plasmid shows the presence of 6 HpaII restriction sites in the DNA fragments located between Sacl and Xbal restriction sites (Fig. 3).

Plasmids obtained from the transformants transfected with AAAF treated c-H-ras, were digested with restriction enzymes (BamHI and Sacl), and subjected to agarose gel electrophoresis (Fig. 4).

All transformants contained c-H-ras sequence. This was evident by the presence of 6.4 and 4.3kb DNA fragments upon BamHI digestion and of 7.0, 2.8 and 0.9kb DNA fragments upon Sacl digestion.

When control and AAAF-treated plasmids were digested with a combination of Sacl and Xbal restriction enzymes, 6.8, 2.0 and 0.8kb DNA fragments were generated (Fig. 5).

When 0.85kb DNA fragments obtained from AAAF-treated plasmids were further digested with HpaII restriction enzyme, 415, 259, 105bps and other small fragments were obtained (Fig. 6A), indicating the absence of the HpaII recognition site (CCGG) at codon 11 and 12.

When the control plasmid (wild type) was ana-

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**Fig. 1.** Silica gel thin layer chromatography of N-acetoxyacetaminofluorene. 
1,2: N-hydroxy-2-acetamidofluorene (AAF) 
3,4: N-acetoxyacetaminofluorene (AAAF)

**Fig. 2.** Silica gel thin layer chromatography of control and AAAF-treated pbc N1 digested with exo- and endonucleases. The pbc N1 and AAAF-treated pbc N1 plasmids were digested with both 0.2 units of spleen phosphodiesterase II and deoxyribonuclease I for 15 min at 37°C and [32P] postlabeled by the method described in the text. The digested DNA were chromatographed on a thin layer silica gel plate using a solvent system of benzene: methanol (9:1), then autoradiographed. The letters indicate the positions of migration of each 3'-phosphate mononucleotide and the arrow indicates the position of dGp-C= (N-AAF).
Fig. 3. The restriction map of c-H-ras oncogene inserted into pBR322 (pbc N1).
The number indicates the size of base pairs and the arrows indicate the positions of restriction sites recognized by the enzymes.

Fig. 4. Agarose gel electrophoresis of plasmids harboring AAF-treated c-H-ras gene.
M. Molecular size marker (λ Hind III)
No. 1-4: AAF-treated plasmids (undigested)
No. 5-8: AAF-treated plasmids (BamHI digest)
No. 9-12: AAF-treated plasmids (Sacl digest)

lyzed by the same method, 415, 204, 105bps and several other fragments were obtained (Fig. 6B), indicating the presence of a Hpal recognition site at codon 11 and 12.
Fig. 5. Agarose gel electrophoresis of the subcloned AAAF-treated c-H-ras gene harboring plasmids digested with restriction enzymes.
M. molecular size marker (λ HindIII)
1. pbc N1 plasmid (control) digested with SacI and Xbal
2. AAAF-treated plasmids (subcloned) digested with SacI
3. AAAF-treated plasmids (subcloned) digested with SacI and Xbal

Fig. 6. Analysis of restriction fragment length polymorphism of AAAF-treated c-H-ras gene.
A. pbc N1 digested with SacI and Xbal
B. Control 850 base pair fragments (SacI and Xbal digestion) digested with HpaII
C. AAAF-treated 850 base pair fragments (SacI and Xbal digestion) digested with HpaII
M. Molecular size marker
Oligonucleotide Hybridization

Selective hybridization of oligonucleotide probes complementary to the normal or mutant alleles of codon 61 of c-H-ras gene with AAAF-treated c-H-ras was done. This demonstrated no hybrid signals with mutant alleles, indicating the absence of a substitution at codon 61 (Fig. 7).

DISCUSSION

Because AAAF was too genotoxic to conserve the plasmids viable at moderate concentration, in the present study the modification of c-H-ras oncogene by AAAF was carried out at a mild reaction condition (<0.1mM AAAF for less than 3min). When the whole plasmids were treated with AAAF at more than 0.1mM concentration, they were not able to be cut by BamHI restriction enzyme nor replicated in E.coli when transfected. Therefore, it is assumed that AAAF may attack on many sites of c-H-ras gene and deteriorate it.

The major AAAF modified base of c-H-ras DNA was identified as dGp-C4 (N2-AAF), which is an available reaction product of dGMP and AAAF in vitro (Fig. 2). The compound has been previously characterized by nuclear magnetic resonance spectra and ultraviolet absorption spectra by Kriek et al. (1967). AAAF, a possible in vivo metabolite of N-
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hydroxy-AAF (Miller et al. 1966), has been shown to react readily with guanine in RNA, DNA and guanosine. AAAF, an ultimate hepatocarcinogen, binds to the C₆ position of guanosine of DNA.

It has been demonstrated that chemical carcinogen exposure to rodents in vivo developed liver tumors that contained c-ras genes activated through a single base substitution at codon 12 or 61 (McMahon et al. 1987). Several cell transformation studies indicated that certain chemicals can induce point mutations at codon 12 and 61 in c-H-ras gene (Skumar et al, 1983; Zarbl et al, 1985; Voussden et al, 1986; Wiseman et al, 1986; Bos 1988). But the predominance of point mutation, which has been detected in vivo at codon 12 did not reflect sequence mediated preferential susceptibility of this site to initial DNA aduction or to replication errors (Marien et al, 1989). The appearance of mutational change at codon 12 in the present study indicates the repair error. However, the mechanisms of mutation are not understood yet because no evidence that selective interaction between guanine nucleotides at codon 12 and AAAF is available. The hybridization of oligonucleotide containing codon 61 mutant alleles of c-H-ras with AAAF treated c-H-ras revealed no substitution at codon 61, indicating that the mutational alteration by AAAF at this site is less potent than that at codon 12.

These results are inconsistent with the results obtained by Voussden et al. (1986) who observed the GC→TA transversion at codon 61 of c-H-ras by AAAF in vitro.

Although we have shown that AAAF modifies mainly the C₆ position of guanine nucleotides in the c-H-ras oncogene and causes the selective mutation at codon 12, the error repair system at this site is still obscure.

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