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P^{53} gene of chang-liver cells (Atcc-Ccl13) exposed to aflatoxin B_1 (Afb): the effect of lysine on mutation at Codon 249 of Exon 7

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Summary
The effect of different regimes of lysine-pre treatment on mutation at the 3rd nucleotide base of codon 249 which is located at the 7th exon of p^{53} gene of Chang-liver cells (CCIL13) exposed to aflatoxin B_1 (AFB_1) has been investigated. There is an indication of inhibition of 1 ug/ml AFB_1 – induced mutation by pretreatment of cells for 72 hr with 5-molar fold lysine equivalent of 1 ug/ml AFB_1 1 u/g ml AFB_1 was the does at which there was 50% survival among the cells of CC13 in cytotoxicity studies.
The results suggest chemo prevention of AFB_1 induced mutation at codon 249 locus of Exon 7 in CCL13's p^{53} gene and by implication, maybe, AFB_1-induced primary liver cancer.

Keywords: *P^{53} Gene, Aflatoxin B_1; Lysine*

Résumé
L'effect de differnets regimes de pre-traitement a la lysine sur la mutation a la 3ieme base nucleotidique du codon 249 qui est localise au 7ieme exon du gene P53 des cellules du fobies de Chang (CCL13) expose a l'aflatoxin B_1 (AFB_1) a ete investigue. II ya une indication de l'inhibition de la mutation induite par l'AFB_1 a 1ug/ml l'or d'im pre-traitement des cellules pendant 72 houres. Avec 5 fois la concentration molaire de lysine equivalente a 1ug/ml d'AFB_1, la dose de 1ug/ML d'AFB_1 de ete la dose a laquelle il ya en 50% de survie parmis les cellules de CC13 dans l'etude cytotoxique. Les resultats suggerent la chimioprevention des mutations induite par l'AFB_1 au niveau du codon 249 du 7ieme exon dans le gene P53 des CC13 et par implication, l'AFB_1 pourrait induire le cancer primaire du foie.

Introduction

A link based on circumstantial evidence has been reported between high exposure to AFB_1 and mutation at the 3rd nucleotide base of codon 249 which is located on the 7th exon of P^{53} gene of cells of primary liver cancer from tropical countries of the world [1,2,3,4,5,6,7].
Treatment of rats with AFB_1 has been reported to result in its binding to liver-DNA and its chromatin proteins [8]. Such a treatment also resulted in its being bound to other proteins such as serum albumin [9]. It has earlier been reported that AFB_1 binds in vitro to other macromolecules [10]. Investigation of AFB_1 binding to DNA [8] which suggests by implication a more long lasting effect. Further studies showed that Histone H1 component of chromatin which is highly rich in lysyl amino acid residues is bound to a greater extent than all other histone proteins in particular as well as other chromatin proteins in general [8].

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A more detail study revealed that AFB_1 binds preferentially to lysyl amino acid residues in proteins [8]. The binding of AFB_1 to histone proteins has significant functional implications
because histones have been reported to be the packaging material for DNA [8] and histone H1 is he most external of the histone proteins in their packaging function [11]. Because of the high content of basic amino acids in histones, it is conjectured that there is a strong electrostatic interaction between then and DNA and that acetylation of the lysyl sites which is involved in this type of interaction reduces the net positive charge of the histones and loosens the bonds between histones and DNA. Acetylation is reported to occur at the amino group of lysly amino acid residues [12] which is the same site of AFB_1 binding [8]. The effect of AFB_1 binding to histone is therefore likely to be similar to that elicited by acetylation, which is he partial loosening of the histone – DNA bond and the consequent degradation of the histone by specific proteases [13]. It is generally accepted that such a partial loosening of the histone – DNA bonds always precedes gene expression. This means that it is most likely that it is the binding of AFB_1 to lysly amino acid residues in histones with the consequent loosening of the histone-DNA bond that makes P^{53} accessible for mutation. It is also likely that the binding of AFB_1 to histones with the consequent loosening of histone is primary to its binding to the DNA of P^{53} genes even though the binding to DNA subsequently exceeds its binding to histones. Also, reports that the binding of AFB_1 to DNA that is responsible for the inhibition of RNA Synthesis [13] which is involved in gene expression. The implication of the above is that it is the binding to chromatin proteins that may be involved in the expression of mutated the P^{53} gene resulting from the interaction of AFB_1 with DNA and chromatin proteins. The P^{53} gene is reported to be mutated in hepatocellar carcinoma after exposure to aflatoxin. It is therefore plausible to conjecture that the presence of lysly group extraneous to the lysly residues on the histone proteins of chromatic would competitively inhit and may be prevent mutation of the P^{53} gene at the target locus of codon 249 at exon 7 of the gene and may in turn result in the chemo prevention of primary liver cancer.
The above is the rationale for the research study reported here.

Materials and methods

Chemicals and reagents: All chemical used of extraction of DNA, lysine, and dimethlsulphoxide (DMSO), Aflatoxin B_1= (AFB_1) and Tripsin-EDTA were obtained from Sigma Chemical CO. (St. Louis, M.O.). ($\wp$ -32P) ATP (4500 Ci/mm) was purchased from Promega Madison WI. Dulbecco's modified Eagle media (DMEM) and the primers used in Polymerase-Chain-

Reaction (PRC) – amplification were obtained from the Institute of Molecular Biology, U.L.C.A.

Cells and in vitro culture conditions: Chang liver human cells (ATCC-CCL13) were obtained from American Type Collection (Rockville, MD). The cells were maintained in a medium comprising 90% Eagle' medium; 10% fetal Bovine serum (FBS). Incubation was in 5% CO_2, 95% air atmosphere.

Cytotoxicity assay: Viable CCL13 cells were harvested by trypsinization in 0.25% tripsin and 0.02% EDTA from confluent culture of cells in 500 mmT-flask. In all, 200 viable cells, based on counts in a hemocytometer using 0.4% trypan blue dye, were seeded in 100-mm dishes. 10 petridishes of culture were used per AFB_1 treatment point (See Fig. 1). The cells were allowed to attache for 3 hrs before being treated with different amounts of AFB_1. A control of ten CCL13 culture plates treated with DMSO, the solvent in which AFB_1 was carried was also set up. After 5 days of incubation of 37 °C in a humidified atmosphere of 5% CO_2, the cells were fixed in methanol and stained in 4% v/v Giemsa and the surviving colones counted. Each point on the curve in Fig. 1 is the mean count from 10 petridishes. Standard deviation of counts were also calculated for the mean of counts.

Dna Extraction Procedure: Approximately 4 x 10^7 trypsinized CCL13 cells from cultures treated as described hereunder were centrifuged at 800 x g for 5 min at room temperature:

(1) cells treated with 1 ug/ml AFB_1, (2) cells treated with same amount AFB_1 for 72 hours before being treated with 5 molar-fold lysine equivalent of AFB_1, (3) cells treated simultaneously with the same amount of AFB_1 and 5 molar-fold lysine equivalent of AFB_1, (4) cells treated with 5 molar-fold lysine equivalent of 1 ug/ml AFB_1, for 72 hrs and (5) cells treated with DMSO. The supernatant of the pellet of cells centrifuged was decanted and cells were resuspended in guanidine isothocyanate buffer (GIT buffer, 4MGIT, 3M sodium acetate and B-mercaptoethanol), which dissolved the cells, releasing the nucleic acids into the buffer. The solution of cells in GIT-buffer was layered over 4 ml of caesium chloride (CsC1) buffer, filled with GIT buffer and centrifuged in Beckamann SW 41 tubes, at 174,000 x g 20 °C overnight. The DNA was pipetted off the CsC1 in the lower third of the tube and purified according to the protocol of Chirgwin *et al.* (1979) [14]. The DNA precipitate was resuspended in Tris/EDTA buffer (TE buffer, 10 mM, pH 7.4 and 0.1 m M, pH 8 EDTA), in which it dissolved completely after several hours. 5 Abait ul of these solutions were made up to 1 ml with distilled water in a spectrophotometer civette and its optical density (OD) read at 260 mm, and 280 mm. The ratio of OD at these two wavelengths was within acceptable range (1.6 – 2.0) which attested to the purity of the DNA extracted.

Fig. 1: Cytoxicity of AFB_1 tp CCL 13 cells pretreated with and not pretreated with 5-fold molar lysine equivalent of AFB_1 only; cells pretreated with 5-fold molar lysine equivalent of AFB_1. Each point on the figure is the mean obtained from counts taken from culture plates treated with DMSO, the solvent in which AFB_1 was carried, was also set up Bars are standard deviations of counts.

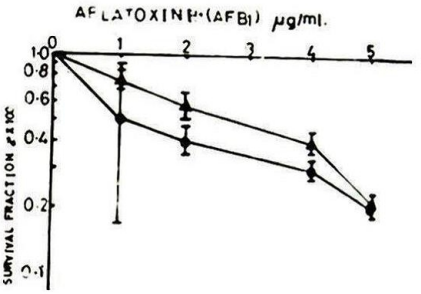

Fig. 1.

Polymerase Chain Reaction (PCR):

Perkin Elmer 960 series PCR machine and Taq polumerase wre used in PCR-amplification of 107 bp DNA segment which encompassed codon 249 at Exon 7 of P^{53} gene DNA extracted from CCL13 cells. The PCR-mix contained the following in a total mix of 100 ul : 500 ug of target DNA, 200 mg of each primer; 5 ul of 5% DMSO; 2 units of Taq polymerase in 0.5 ul of buffer, a balanced mixture of deoxynucleotide triphospahates comprising 100 uM of each dATP, dCTP, and dTTP, and 10 ul of PCR buffer (perkin Elmer Cetus – 50 mM KCl, 10 mM Tris-C1 PH 8.3; 15 mM $MgCl_2$; 0.01% w/v gelatin). The mix was made up to 100 ul by double distilled water. The constituents of the mix were thogoughtly mixed in microcentrifuge tubes by rapid repeated pipetting and discharging of mixture through pipette tips of 200 u 14 capacity Eppendor piettors. The mix was finally overlaid by a few drops of sterile mineral oil and put into prevarmed Perkin-Elmer Cetus thermal cycler programmed for 35 cycles. The primers used have the sequences indicated as follows:

(1) 5-CTGGAGTCTTCCAGTGTCAT -3
(2) 5-CTTGGCTCTGACTGTACCAC-3

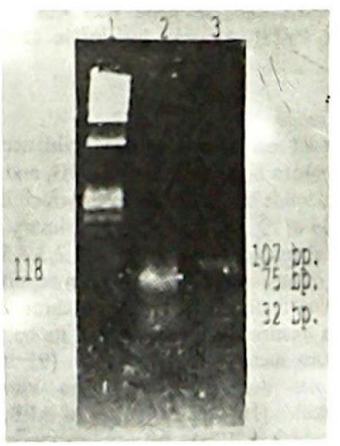

Fig. 2: Electrophresis in 2.5% Nusieve gel of Hae III restriction of 107 bp PCR amplification product of segment of P53 gene DNA encompassing codon 249 of untreated CCL 13 cells. Lane 2 shows Hae III restricted PCR-product fragments of 72 and 35 bp, Lane 1 is standard of 0 X 174 RF DNA/Hae III fragments. Lane 3 is unrestricted PCR product of 107 bp. If any mutation was instrinsically present at codon 249 (AGG) of P^{53} gene of CCL 13 cells, Hae III restriction site between this codon and codon 250 (CCC) (GGCC) would not have been present.

Electrophoresis: Electrophores depicted in Figs. 2 and 3 were carried out on 2.5% a gar- Nusieve in TBE buffer (Tris base 0.05 M, Boric acid 0.05 M, EDTA-Na_2. $2H_20$ 1 mM = 1 litre double distilled water). 10 ul of Ethidium bromide was added to 100 ml of melted Nuseive in TBE poured on electrophoresis tray, and allowed to solidify for 45 minutes. PCR-products of 107 bp segment of DNA from CCL13 restricted and unrestricted with Hae III (see Fig. 2) were loaded on gel and run at 100 volts. Electrophoresis on Fig. 3 depicts 107 bp segment of DNA from different regimes of treatment of CCL13 with AFB_1 and lysine. Electrophoresis on Fig. 4 depicts Hae III restricted PCR-products in which one of the primers was labelled in a reaction mixture containing 2 ul. X 10 kinase B buffer, 1.5ul T_4 kinase (specific activity 2 units/0.5 ul); 5 ul. ($\wp$ - ^{32}P) ATP and 11.5 ul double distilled water. The resultant digest from different regimes of treatment with AFB_1 and lysine were run on 30% bis-acrylamide (14 ml bis-acrylamide, 30.5 ml H_20, 5 ml TBE x 10, + 10, + 1 ml of a solution of 100mg ammonium persulphate in 3ml H_2 0 + 30 ul of N, N, N, N-tetramethylethylendediamme-(TEMED).
Electrophorresis was run in 1 x TBE buffer at 100 volts.

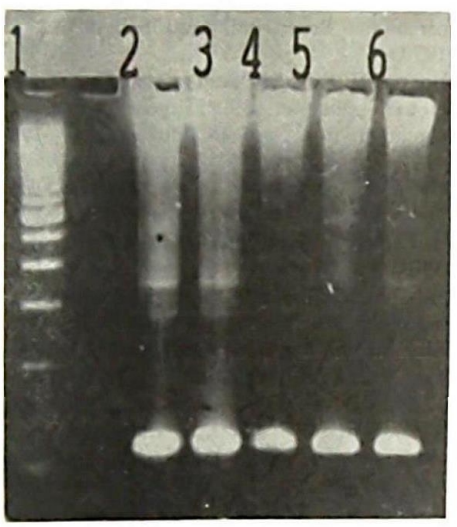

Fig. 3: Electrophotesis on 2.5% Nusieve agarose gel of PCR-product of 107 bp segment of DNA which spans codon 249 at exon 7 of CCL 13's P^{53} gene treated with different regimens of AFB_1 lysine combination indicated below. Lane 1 is standard 100 bp DNA ladder, catalogue number – 56285X – Gibco BRL; Lane 2-PCR-product from cells treated with 1 ug/ml AFB_1; Lane 4-PCR product from cells treated with 5 fold-molar lysine equivalent of 1ug/ml AFB_1; Lane 5-PCR product from cells treated with 5 fold molar lysine equivalent of 1ug/ml AFB_1; Lane 6-PCR-products from cells treated with DMSO.

Sequencing pcr-product: The PCR-product of untreated CCL13 cells was sequenced to ascertain that sequence at codon 249 of exon 7 53 gene of CCL13 was AGG as in the wild type of codon 249. The standard dideoxynucleotide sequencing protocols of Sanger *et al* (1977) [15] with modifications involving the substitutionof sequenase (U.S.B.) for Klenow fragment of DNA polymerase I was used. Primer was labelled with ($\wp$ - ^{32}P) ATP as described under 2.6 and used.

Result

Cytotoxicity: Only 1 ug 1 ml of AFB_1 killed 50% of CCL13 cells whereas in cells pretreated for 72 hrs with 5 fold-molar lysine an equivalent of AFB_1 prior to AFB_1 exposure required 4 ug/ml AFB_1 to effect the same 50% kill (see Fig. 1) Abait 5 ug/ml AFB_1 was essentially cytotoxic to the same extent in lysine pretreated and untreated CCL13 cells.

Electrophoresis in 2.5% Agar-Nusieve: PCR-amplification products of DNA segment encompassing codon 249 at Exon 7 of p^{53} gene-DNA extracted from CCL13 cells exposed to different regimes of AFB_1 and lysine treatment are shown in Fig. 2. Lane is the 100 bp DNA-ladder-standard obtained from GIBCO BRL (Cat No. 56285X). The PCR product is a 107 bp DNA fragment. Fig. 2 is an electrophoresis of Hae III restriction products of 107 bp of PCR-amplification-product of DNA which encompasses codon 249 of Exon 7 of untreated CCL13 P^{53} gene. The result which shows that 107 bp PCR amplification product is susceptible to Hae III restriction, confirms that the nucleotide sequence at codon 249 of CCL13 is most likely AGG as in the wild type P^{53} gene. The specific restriction site of Hae III is between codon 249 and 250, i.e. between the sequence AGG of codon 249 and CCC of codon 250 (AGG CCC). The standard on lane 1 of Fig. 3 is OX 174 RF DNA/Hae III.

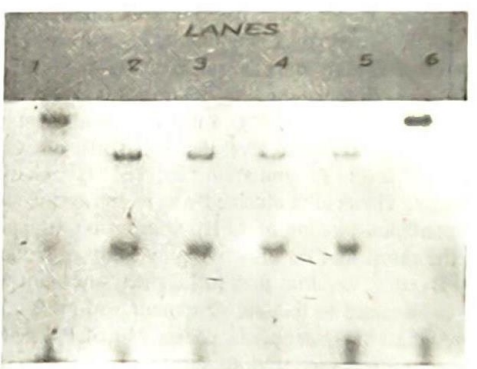

Fig. 4: Electrophoreis on 30% Bis-acrylamide gel of Hae III restricted PCR-amplification product of 107 bp DNA labelled with (^{32}P) ATP-labelled primer. 50 ng of Hae III restricted PCR amplification products from each Lysine/AFB_1 for 72h before being exposed to 1 ug AFB_1 Lane 3 – cells treated simultaneously with 1 ug/ml AFB_1. Lane 1 – cells treated with 5 fold-molar lysine equivalent of 1ug/ml. AFB_1 Lane 6 – unrestricted PCR product from DMSO treated CCL13 cells.

Electrophresis: Hae III Restriction Products in 30% Bic-acrylamide: Fig 4 shows the result obtained with Hae III restriction products of 107 bp DNA PCR amplification products from CCL13 cells labelled with ($\wp$32P)-ATP and subjected to different regimes of AFB_1 and lysine combination treatments as indicated in Fig. 4. Lane is the 107 bp DNA ($\wp$ 32P) ATP - labelled unrestricted PCR products of untreated CCL13 cells.

The unrestricted fraction of PRC products in lanes are indicative of mutations at the third base "G" of codon 249 – AGG by treatment. Any mutation on third base of codon 249 alters Hae III specific tetrsa base restriction site of GG CC between codons 249 (AGG) and codon 250 (CCC).

Discussion

The results in Fig. 2 of Hae III restriction analysis of PCR-products of untreated CCL13 indicates that the sequence at codon 249 is as in the wild type of P^{53} gene. This was subsequently confirmed by the result obtained in the sequencing of a sample of PCR product. These results gave the required clearance for the use of CCL13 for the current study reported here. The cytotoxicity results showed at least a four-fold inhibition of cytotoxicity of AFB_1 CCL13 cells by lysine at 50% - kill of cells (Fig. 1). This was likely to be as a direct result of competitive binding of AFB_1 of lysine making less of it available for cytotoexity of CCL13 cell. AFB_1 has been reported to bind preferentially to lysly residues in proteins in vivo [8].

The result obtained in the Hae III restriction analysis of (γ -32P) ATP-labelled PCR-products obtained from CCL13 cells treated with different regimes of AFB_1 and lysien indicated that pretreatment of cells with lysine 72 hrs before exposure to AFB_1 prevented mutation at codon 249, as can be seen in lanes 5,4,3, and 2 of Fig. 4 when compared with lane 6 for CCL13 cells untreated and lane 1 for cells treated with AFB_1 only. It also appears that simultaneously treatment of cells with AFB_1 and lysine (Lane 4) and treatment with lysine before treatment with AFB_1 - (Lane 2) elicited the same effect. It must be acknowledged that the result on which the above conclusions were based were qualitative. There is therefore a need for a quantitative comparison of the levels of mutation in cells subjected to the different AFB_1-lysine combination – regime – treatments. It would be observed in lane 1 of Fig. 4 that even with treatment with AFB_1 at a treatment level that killed 50% of CCL13 cells, the level of mutation induced is relatively significant. The results obtained was in consonance with the preferential binding of AFB_1 with lysine. It appears from the result that for effective prevention of mutation by AFB_1, it is required that sufficient lysine molecules must be in place at the site of mutation of AFB_1. It is obvious that AFB_1 access to codon 249 of P^{53} gene is necessary for mutation to occur and this is likely to occur when AFB_1 binds to lyslyl residues in histones and the DNA. The partial loosening of the histone-DNA bond by AFB_1 binding to histone was expected just as occurs when lysly amino acid residues in histones were acetylated [13]. The presence of lysly groups extraneous to the ones in histones prevents this loosening out of the histone-DNA electrostatic bond and consequently prevented accessibility of AFB_1 to mutation site on codon 249 of the P53 gene of CCL13 cells. May be this accessibility would be more in the setting of integration of hepatitis B virus (HBV) in the map position P12 – P112 on chromoseom 17. It has been reported that Pre S2/S region of integrated hepatitis B virus DNA encoded a transcriptional transactivator [15]. Hepatitis B virus gene products have also been reported to interact with DNA repair proteins [16]. The presence of HVB viral DNA at a position on chromosome 17 contiguous to P53 gene is likely to have the reported transcriptional transactivation on the P53 gene and interaction with DNA repair proteins with the consequent increase in its expression and therefore more accessibility to AFB_1 mutation when test cells are exposed to AFB_1 and post-transcriptional downregulation of gluthahione transferase a in HBV transfected Hep G2 cells [17].

The effect of the presence of integrated HBV-DNA in liver cells to mutation at codon 249 of P53 gene by AFB_1 needs to be investigated in more details. The review article by Lasky and Magder (1997) [18] did not quit address this problem.

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