CHAPTER – IV

VD₃ INHIBITS 2-AAF-INDUCED DNA CHAIN BREAKS AND DNA-PROTEIN CROSSLINKS DURING THE EARLY STAGES OF NEOPLASTIC TRANSFORMATION IN RAT LIVER
Introduction

Cancer is a genetic disease ensuing from cells accumulating mutations in critical growth regulatory genes, including oncogenes and tumor suppressor genes (Sherr, et al, 2004). In many human cancers, the tumorigenic process is believed to be fueled by global genomic destabilization that facilitates the activation of oncogenes and the inactivation of tumor suppressor genes. The role that compromising the general maintenance of genome integrity plays in promoting cancer has been appreciated in large part through the studies of human hereditary cancer syndromes associated with genetic instability, as well as through the generation and analysis of mouse knockouts (Levitt et al, 2002; Deng, et al, 2003). Proteins encoded by different genes are central in the response of cells to DNA damage, acting either to implantation checkpoint responses that allow cells to arrest in the cell cycle, or alternatively, to directly execute the repair of DNA lesions (Artandi, et al, 1997).

The essential nature of genes involved in maintaining genomic integrity reflects the fact that cells are constantly being exposed to DNA damage. Damage to DNA can occur through any of a number of different mechanisms, including the induction of mismatches, cross links or strand breaks. Particularly deleterious are double strand breaks (DSBs) in DNA (Jackson et al, 2002), which are difficult to repair due to the propensity of the free ends to separate from each other in the cell. Moreover, if not repaired correctly, these lesions are particularly mutagenic: broken DNA ends can recombine inappropriately with other sites in the genome, thereby driving gross chromosomal instability, in the form of translocations as well as gene copy number changes. DSBs in cells arise as a result of exposure to external agents or endogenous cellular processes (Khanna et al, 2001). Exogenous inducers of DSBs include reactive oxygen species generated during normal cellular metabolism, stalling of replication forks during DNA replication due to single strand DNA breaks or other lesions, and defects in DNA repair protein function.

The optimal response of the cell to DSBs is to attempt to repair such DNA lesions, which at the same time would protect the cellular genome and preserve the viability of
the cell. Two classes of proteins act to implement repair, proteins involved in checkpoint activation and proteins involved directly in repair (Van Gent, et al, 2001). The checkpoint responses, invoked during the G1, S, and G2 phases of the cell cycle, are thought to exist to allow cells adequate opportunity to halt and repair their DNA before progression through the cell cycle. The G1 and S phase checkpoints specifically prevent the replication of damaged DNA, while the G2 checkpoints prevent the segregation of broken chromosomes during mitosis. In addition, the cell has two major pathways to affect the repair of DNA DSBs. One is the non-homologous end joining (NHEJ) pathway through which double strand breaks are ligated without the use of a template, and which predominates in the G1 phase of the cell cycle. Alternatively, homologous recombination (HR) allows repair of DSBs based on the sequence of the sister chromatid, and this process occurs in S or G2. Cells defective in checkpoint responses or either of these principal DNA DSB repair pathways accrue DSBs and are susceptible to gross chromosomal instability (Artandi, et al, 2000).

The induction of an apoptotic response by DNA damage frequently occurs through the activation of the p53 tumor suppressor protein (Vousden, et al, 2002). The p53 is the most commonly mutated gene in human cancer, a finding that reflects its crucial anticancer activity (Levine, et al, 1997). The p53 acts to obstruct tumorigenesis by serving as a cellular stresses, including DNA damage, hypoxia, or proliferative signals, p53 protein becomes stabilized, causing cells to undergo either cell cycle arrest or apoptosis (Vousden, et al, 2002). The cell cycle arrest response is a checkpoint function that allows cells to pause in the cell cycle either temporarily or permanently to prevent the perpetuation of potentially oncogenic mutations. The apoptotic response is one that acts to cull cells that are proliferating aberrantly or that have suffered DNA damage, such as through checkpoint or repair defects. Cells with such lesions and which also lack p53 proliferate or survive inappropriately, propelling the development cancer.

Cancer has been referred to as a DNA-mediated degenerative disease which itself has a genetics (German, 1985). At the cellular level, cancer can be considered as a genetic disturbance because critical events in tumorigenesis include alterations of cellular DNA. Although the nature and order of appearance of such alteration is not fully
understood for any type of neoplastic, their cumulative number is considered crucial for the expression of the transformed phenotype (Bishop, 1991). The transformed phenotype bounds in structural and functional alterations that are thought to be acquired independently during the development of the tumor.

The propensity of cancer cells to show multiple gene mutations, DNA lesions and karyotypic abnormalities underscores the concept that multistep carcinogenesis progresses by the accumulation of discrete genetic alterations. Central to understanding the cause and effect relationships between the many endpoints associated with neoplasia is knowledge of how this genetic change occurs and what proliferative advantages is bestowed on the cell. Accumulating evidence suggests that genomic instability may provide the driving force behind the genetic plasticity characteristic of cancer cells (Nowell, 1976; Loeb, 1991; Selvanayazam et al, 1995). Genomic instability includes gene mutation, gene amplification, chromosomal destabilization, and cellular transformation (Morgan et al, 1996). Successful application can lead to mutations, many of which result from gross chromosomal change. Indeed, the preponderance of chromosome arrangements identified in neoplasms serves to emphasise the importance of studying processes contributing to the formation of chromosomal rearrangements (Mitelman, 1991; Soloman et al, 1991).

It was ascertained during the 1950’s that malignant exudates, because of their advanced stage of cancer growth, showed deviating karyotypes (Hansen-Melender et al, 1956; Ising and Levan, 1957). Studies on chromosomal changes carried out during the advanced stages of various forms of ascites tumor growth, have also been reported to show abnormal karyotypes (Levon et al, 1977). On a population basis, increased frequencies of aberrations are an identification of exposure a factor that increases the risk of cancer and genetic diseases. Cytogenetics has thus become an invaluable tool in the diagnosis and clinical management of certain types of cancer (Testa, 1991).

Cellular DNA is generally considered to be the most critical cellular target when considering the lethal carcinogenic and mutagenic effects of drugs, radiation and environmental chemicals (Birnboin and Jercak, 1981). These agents may damage DNA
by altering bases or disrupting the sugar phosphate backbone. Oxygen radical may attack DNA at either the sugar or the base, giving rise to a large number of products (Hutchinson, 1985). Attack at a sugar ultimately leads to sugar fragmentation, base loss, and a strand break with a terminal sugar residue. Such single strand breakage occurs in bacteria and mammalian cells when exposure to $\text{H}_2\text{O}_2$, $\text{O}_2$, gamma radiation or ozone (Ananthaswamy and Eisenstark, 1977; Birnboin and Kanabus-Kaminska, 1985). Although base damage may have serious consequences for a cell, low levels of base damage are difficult to measure by physical or chemical means (Paterson, 1978). Earlier methods for detecting DNA unwinding in alkali have required physical separation of single- from double strand DNA using a hydroxypeptide column, specific nuclease digestion and precipitation or filter binding (Ahnstrom and Erixon, 1973; Kohn and Ewig, 1973; Rydberg, 1975). Moreover radiolabelling of cells was required for detection of the small amounts of DNA involved. In cells where radiolabelling was not feasible, sensitive fluorimetric methods were substituted to permit detection and quantification of DNA after column or filter separation (Bradley et al, 1978; Kanter and Schwartz, 1979; Erickson et al, 1980). In this study, we have used a technique of fluorimetric analysis of DNA unwinding (FADU) using a fluorescent dye for minority DNA unwinding, according to our method (Sarkar et al. 1997). In this chapter we report the effect of $\text{VD}_3$ in the transformation of neoplastic cells in rat liver treated with 2-AAF and ability the generation of DNA-strand breaks and protein cross linking as well.

**Materials and methods**

**Animals**

Male Sprague-Dawley rats 80-100g body weight, obtained from Indian Institute of Chemical Biology (CSIR), Kolkata. The animals were acclimatized to standard laboratory conditions (Temperature 24±1°C, RH 55±5% with the altering 12:12 h light-dark cycle) in Tarson cages (4 to 6 rats per cage) for one week before the commencement of the experiment. During the entire period of study, the rats were fed with a semi-purified basal diet (Lipton India Ltd., Mumbai, India) and water ad libitum.
All animals shall, strictly be maintained according to the NIH guidelines of care and use of Laboratory animals (CPCSEA Regn. No:-367/01/C/CPCSEA)

**Experimental regimen**

Rats were divided into four groups (A-D) as described in chapter-III. In groups C (2AAF control) and D (2AAF+ VD3), hepato-carcinogenesis was initiated by chromic feeding of the rats with (0.05% in basal diet), 5 days a week on and from week 4. Group A was the normal vehicle control. Group B (VD3 control) and D rats received 0.03μg/100ml in propylene glycol, per OS, twice a week starting 4 weeks prior to 2AAF administration and continued for entire length of the study i.e., for 20 consecutive weeks. Two different sets of animals were maintained for estimation of DNA strand break and DNA-protein cross link (DPC). For estimation DNA strand break study hepatic DNA from all of the above groups was isolated at 18, 20h after the last 2AAF feeding at 45. For estimation of DPC, the second set of rats from all the groups was killed at five different time intervals, i.e., after 3,6,12,24 and 48h following the last feeding of 2AAF at day 45; livers were promptly excised and hepatic DPC was isolated.

**Isolation of DNA**

A slight modification method of Gupta (1984) was utilized for the isolation of DNA from rat liver. In this procedure, the frozen tissue (5g) was suspended in 3.0ml of 1% sodium dodecyl sulphate, 1mM EDTA, 10mM Tris-HCl buffer (pH 7.4) and homogenized in a Teflon-coated homogenizer for 30 sec. The homogenate was incubated at 37°C with ribonuclease-A at a concentration of 200microgram/ml for 1h following the overnight treatment with proteinase K (500microgram/ml) at 55°C. The solution was extracted successively with one volume each of phenol, 1:1 mixture of phenol-sevag (chloroform-isoamyl alcohol, 24:1v/v) and sevag as described (Gupta, 1984). DNA in the aqueous phase was then precipitated by the addition of one-tenth volume of 5M sodium chloride and two volumes of cold ethanol, collected by centrifugation at 13,000 X g for 5 min, washed twice with 3ml of 70% ethanol, and
dissolved in 0.5ml of TE buffer (20mM Tris, 1mM EDTA, pH 8.0). The concentration of DNA was estimated spectrometrically. (Reddy et al, 1984) and the solution was stored at -20°C.

**DNA Unwinding Assay**

The principle of this assay is that the fluorescent dye ethidium bromide (EtBr) binds selectively with double-stranded DNA in presence of single-stranded DNA when short duplex regions in single-stranded DNA molecules are destabilized by alkali treatment (Morgan and Pullyblank, 1974).

After isolation of DNA from all the groups, the solution was then divided among three sets of tubes equally. The contribution of fluorescence by the components other than double stranded DNA (including free dye) is estimated from a blank sample (B) in which the DNA solution is highly sonicated at first and then treated with alkali under conditions that cause complete unwinding of low-molecular weight double–stranded DNA. A second sample is used for estimation the total fluorescence (T) meaning the fluorescence due to the presence of double-stranded DNA with contaminants. The difference of these two i.e., (T – B) provides an estimate of the amount of double stranded DNA i.e., the DNA pool. A third sample (P) was exposed to alkali conditions sufficient to permit partial unwinding of the DNA, the degree of unwinding being related to the size of the DNA. The fluorescence of the sample was less than that of the blank i.e., (P – B) provides an estimate of the amount of double-stranded DNA remaining. Percent D is given by the following equation

\[
\text{Percent D} = \frac{(P - B)}{(T - B)} \times 100.
\]
**Estimation of Single-Strand Breaks**

It seems that the distribution of single strand breaks in the DNA population follows a simple Poisson’s law. Under these circumstances, it is possible to make an approximate estimate of the average number of the single strand breaks (n) per DNA unit from the simple equation (Basak, 1996):

\[ e^{-n} = \frac{D}{S + D} \]

Where, S represents percentage of DNA that remains single stranded after alkali treatment, D is the percentage remaining as duplex DNA and \( \frac{D}{S + D} \) represents the fraction \( f_0 \) of the molecules without strand breaks. The values of ‘N’ corresponding to different DNA solutions from different groups were then estimated.

**Alkali Treatment, Neutralization and Shearing of DNA**

The optical density (O.D.) of the DNA solution was adjusted to 2.0 at 260nm. For alkali treatment (to denature), 2.0ml of DNA solution in TE buffer (2mM Tris, 1mM EDTA, pH 8.0) was mixed with an appropriate aliquot (about 2.4ml) of alkali solution (0.1N sodium hydroxide, 0.001M EDTA) so that the pH of the mixture becomes 12.8. About 10 min after that (determined by trial experiments), the pH of the mixture was further brought down to about 9.0 by the addition of an approximate aliquot (about 1.3ml) of an acid solution (0.025m Tris, 0.225N HCl). The DNA was then sheared by passing the DNA solution (20 -25 times) through a needle of 24 gauge using a hypodermic syringe.

**Fluorimetric Analysis of DNA Unwinding (FADU)**

After the isolation of DNA from all the groups, the purity of the DNA solution was checked by determining the ratio of absorbance A260 / A280 and A260 / A230. The solution of DNA was distributed into 12 test tubes containing 2.0ml of DNA solution having O.D. equal to 2.0 at 260nm. The tubes were designated as T, P or B in each group. Initially, the DNA solution in the tube B was sheared. To P and B tubes, first
alkali solution was added, mixed and then the tubes were incubated at 15°C for 10min. Denaturing was stopped by chilling at 0°C and addition of acid solution i.e., the denaturing and the neutralizing solutions were mixed together before and addition of DNA solution. An aliquot of EtBr solution (0.2 µl) in 0.003M sodium hydroxide containing 96 µg/ml EtBr was added to each tube and the fluorescence was read at room temperature in a Waters Spectrofluorimeter (excitation at 525nm and emission at 591nm). The extent of DNA unwinding after a given time exposure to alkali is calculated from the fluorescence values of T,P and B samples. The percentage of D (double stranded DNA) is represented by (P-B) / (T-B) x 100.

**Statistical Analysis**

The experimental results have been expressed as the mean ± standard error of mean (S.E.M). Significance was evaluated statistically by the Student’s ‘t’-test (Woodson, 1987). P<0.05 imply significance. Percent inhibition was calculated by using the formula [(mean control – mean treatment) / mean control] x 100.

**Results**

**Effect of VD₃ on 2-AAF induced hepatic DNA-strand break**

A significant rise in the total percentage of hepatic DNA-strand breaks could be observed in 2-AAF control (group-C) rats was 3 fold less (P <0.001) than in normal control rats (group-A) where as the total percentage of aberrant single-strand regions in group-C rats were more than 15 fold higher (P <0.001) than that of normal controls (group-A). The increase in the percentage of SS-DNA is taken as a measure of DNA-single strand breaks. This is actually indicative of the direct DNA-damaging potential of the hepatocarcinogen 2-AAF. In 2-AAF (group-C) a significant increase (P <0.001) in the average number of SS-DNA break / DNA was observed at 18-20 h after 2-AAF feeding on day 45 when compared to the normal control (group-A) (Table 4). However treatment with VD₃ shows, a maximum protective effect i.e., decreased the percentage
of generation of SS-DNA break / DNA (56.39%, P <0.001) when compared to 2-AAF control (group-C).

**Effect of VD$_3$ on the formation of DPC**

Table 4 shows, the DPC coefficients in different groups of rats treated with 2-AAF and VD$_3$. It is evident that, there was a significant induction of DPC following 2-AAF feeding to group-C rats killed at all the specified time intervals, but the amount of DPC formation was maximum (P <0.0001; 66.49 %) after 6h from the last feeding of 2-AAF when compared to normal control rats (group-A). however, the amount of DPC gradually started to reduce after 24h (P <0.01) and came down almost to the basal level after 48h in 2–AAF control rats suggesting a possible involvement of DNA repair mechanism. In VD$_3$ supplemented rats (group-D, the amount of DPC following carcinogen challenge was also increased but to a lesser extent than that of group-C. VD$_3$ supplementation significantly reduced (P <0.001) the formation of DPC in group-D rats on and from 3 h following 2-AAF challenge. There was a gradual reduction (28.64% at 6h... 4.86% at 48h) in the DPC coefficient in group-D rats at all the time intervals, when compared to 2-AAf control rats.
Table 4: Effect of VD₃ on the generation of DNA single-strand breaks and DPC Coefficient in rat liver following 2-AAF challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment(s)</th>
<th>DS DNA [n=10] [Mean%SE]</th>
<th>SS DNA [n=10] [Mean%SE]</th>
<th>Average No Of SS breaks/DNA unit</th>
<th>Inhibition (%)</th>
<th>DPC Coefficient (%DPC/%DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal Control</td>
<td>95.56±0.31</td>
<td>04.44±0.31</td>
<td>0.052±0.03</td>
<td>--</td>
<td>8.23±0.56</td>
</tr>
<tr>
<td>B</td>
<td>VD Control</td>
<td>92.34±0.36</td>
<td>07.66±0.36</td>
<td>0.06±0.03</td>
<td>--</td>
<td>7.94±0.22</td>
</tr>
<tr>
<td>C</td>
<td>2-AAF control</td>
<td>35.67±0.41</td>
<td>64.33±0.41</td>
<td>1.33±0.05</td>
<td>--</td>
<td>66.49±3.76d</td>
</tr>
<tr>
<td>D</td>
<td>VD₃+2-AAF</td>
<td>59.03±0.33b</td>
<td>32.14±2.36b</td>
<td>0.58±0.23</td>
<td>56.39</td>
<td>41.18±2.12c</td>
</tr>
</tbody>
</table>

*Values are Mean ± S.E.(n=10)

aP<0.001 when compared to Normal control (Group A)
bP<0.001 when compared to 2-AAF control (Group C)
cP<0.01 when compared to 2-AAF control (Group C)
dP<0.0001 when compared to Normal control (Group A)
Discussion

Carcinogen-induced DNA damage, DNA repair and SCEs are similar major events during the initial stages of carcinogenesis (Popescu et al., 1984). DNA strand breaks responsible for chromosome damage are generated from DNA base lesions induced by most chemical mutagens. These DNA base lesions are generally repaired by the excision-repair system (Friedberg et al., 1982). The substantial decrement of the single strand breaks can explain one possible mechanisms of the anticlastogenic potential of VD$_3$. One hypothesis is that the $in$ $vivo$ protective effect of VD$_3$, may be due to the excision repair activity. DNA double-strand breaks (DDBs) are generated from mutagen induced DNA lesions in the S phase of the cell cycle and repaired by post replicational repair in the G$_2$ phase and that unrepaired DDBs result in breakage type CAs (Kihlman et al., 1982). The two possible mechanisms are: first, by preventing damage to the chromosome through its antioxidant efficacy, and second, by preventing the DNA breaks by acting on the antiproliferative and differentiation-inducing genes. In $in$ $vitro$ VD$_3$ delays the cell cycle and can thus prolong the repair mechanism. The principal block in cell cycle progression in VD$_3$-treated human T lymphocytes cells occurs in the G$_1$ phase (Rigby et al., 1985). The cyclin-dependent kinase inhibitors p21$^{(Waf1)}$ and p27$^{(Kipl)}$ have been reported to be involved in this process. The p21$^{(Waf1)}$ gene contains a VD$_3$ response element within its promoter region and its expression of p27$^{(Kipl)}$ is markedly induced in certain cancer cell types (e.g. myeloid leukemia and prostate cancer cells) after this exposure to VD$_3$ $in$ $vitro$ (Hengst and Reed, 1996; Wang et al., 1996). Earlier study from our laboratory have established that VD$_3$ can effectively reduce the incidence of CAs in Dalton’s Lymphoma mice (Sardar et al., 1996b). In this Context, the suppression of break type abbreviations by VD$_3$ may be due to modifications of the capability of the post replicational repair of DDBs. Several reports suggests that antioxidants suppress the elastogenic action of tumor promoters and carcinogenesis (Cerutti, 1985). The possible anticlastogenic potentials of VD$_3$ have been neglected this far as has been its antioxidants potentials. In this chapter we have shown that the occurrence of DDBs is greatly reduced by exploring the inhibitory effect of VD$_3$ as antineoplastic agent. VD$_3$ may be effective in offering production by
antioxidant efficacy and preventing the breaks at DNA by acting on the antiproliferative and Differentiation inducing genes.