CHAPTER – III

SUPPLEMENTATION OF VD₃ MODULATES THE EXPRESSIONS OF GST-P AND P53 DURING 2-AAF INDUCED RAT HEPATOCARCINOGENESIS


Introduction

Hepatocarcinogenesis induced by various carcinogens is a multistep and complex process and is a favored model in the rat that facilitates the study of the mechanism of transformation from a normal cell to a malignancy (Farber and Cameron, 1980). The liver is often the first organ to be affected by metastasizing cancer (Elizabeth M et al, 2000). The initiation of hepatocytes to give rise to foci of altered gene expression by genotoxic carcinogens such as the placental form of glutathione S- transferase, (GST-P), has been well documented (Ito et al, 1989). GST-P is commonly used as a method for the identification, quantitation and assessment of rat liver cancer development (Pitot et al, 1990).

Almost all types of primary liver tumors known to occur in humans can be produced chemically in laboratory animals especially in rats (Stewart, 1975). Rat liver is an organ with an especial sensitivity of developing tumors after exposure to many chemicals and drugs (Pitot, 1988; Maronpot et al, 1989). The initiation of hepatocytes by genotoxic carcinogen to give rise to foci of altered gene expression, such as placental isozyme of glutathione S-transferase (PGST*) has been well documented (Imaida et al, 1989) and is commonly used as method of monitoring the development of putative preneoplastic lesions in the liver (Ito et al, 1988). 2-Acetyl amino fluorine (2AAF) is representative of a complete carcinogen for the liver (Goldwarty and Pitot, 1985). AAF (Pitot et al 1987; Hendrich et al, 1987) has different action in rats subjected to a partial hepatoctomy. 2AAF forms different types of adducts in the liver and induced hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic, enzyme-altered focal lesions (Singer and Crunberger, 1984, Saffhill et al, 1985).

In the 2AAF model rat hepatocarcinogenesis PGST* has been described as the most effective single marker of hepatic preneoplastic for most regimens (Hendrich et al 1987, Tatematsu et al 1985). The initiation stage of cancer development is affected in the rat liver by the administration of 2AAF during a period of enhanced cell proliferation induced by hepatocellular necrosis (Solt & Farber 1976, Ito et al 1988). The molecular and cellular processes of initiation of chemical hepatocarcinogenesis of
experimental animals require elucidation (Farber, et al, 1980; Bannasch, et al, 1984; Pitot et al, 1985). Isozymic alteration of Phase II detoxification enzymes, such as epoxide hydrolase’s, glutathione S-transferase (GST) and UDP-glucuronyltransferase, is a biochemical feature of preneoplasia and neoplasia (Lubdahl et al, 1976; Levin, et al, 1978; Satoh, et al, 1985). Although several aspects of these changes remain to be characterized, these markers may be useful for elucidating the mechanism of initiation. Of the preneoplastic marker enzymes, rat GST P-form (GST-P) is markedly and specifically inducible in preneoplastic foci (Satoh, et al, 1985; Tatematsu, et al., 1985; Sato, et al, 1989). Therefore, this enzyme has been used in both the basic analysis of carcinogenesis in experimental animals and in screening for carcinogens and carcinogenic modifiers (Sato, et al, 1989; Ito, et al, 1996).

However the physiological significance of the specific expression of GST-P in preneoplastic cell populations, why it is inducible by so many carcinogens, whether or not the GST-P positive single cell populations are precursors of preneoplastic foci and what is the genetic change(s), if any, are matters of great interest. GST-P belongs to the pi class of GST, a family of multifunctional enzymes that catalyze GSH conjugation of a wide variety of exogenous and endogenous compounds as well as acting as a binding protein of organic anions and carcinogens (Litwack, et al, 1971; Mannervik, et al, 1988). Despite extensive studies, the substrate specificity of GST-P remains unclear as it shows low and broad enzymatic activities against various compounds except for some (Mannervik, et al, 1988; Berhane, et al, 1994; Satoh, et al, 1998).

On the basis of its utility as a marker of all stages of rat liver cancer (Satoh et al, 1985; Ito et al, 1988; Sato et al, 1984) and its efficiency for the detection of 2AAF- induced altered hepatic foci (AHF) ( Hendrich et al, 1987; Ito et al,1988), expression of PGST* may the initiation of hepatocytes in rat liver carcinogenesis. The function of PGST* is abundant in the cell of hepatocytic nodules and hepatocellular carcinoma, is unknown although the fact that it is multifunctional enzymes involved in the detoxification of electrophilic drugs and carcinogens would indicate a crucial role in the mechanism of resistance (Tsuchida and Sato, 1992). Recently, it has been suggested that single hepatocytes that express PGST* may be one population of initiated cells in the liver
Additionally, several investigators have detected PGST\(^*\) single hepatocytes and altered hepatic foci (AHF) in the livers of untreated control rat (Mitaka and Tsukada, 1987; Ward and Henneman, 1990). This finding is in concert with the spontaneously occurrence of initiation (Pitot et al, 1989). Some of the single hepatocytes expressing PGST\(^*\) develop into AHF in the presence of promoting agents (Yokota et al, 1990). These factors imply that some initiated hepatocytes, indistinguishable in hematoxylin and eosin-stained sections from one another and from the surrounding uninitiated hepatocytes, may be detected on the basis of their expression of PGST\(^*\). Since the PGST\(^*\) has been reported to be the best single marker of rat hepatocarcinogenesis (Hendrich et al, 1987; Tatamatsu et al, 1985).

The rat glutathione S-transferase P (GST-P) (Sato, et al, 1989) gene, belonged to pi class GST, is strongly and specifically expressed during chemical hepatocarcinogenesis, and is considered to be excellent tumor marker. The levels of human GST-pi have been significantly increased in a number of tumors including colon, stomach, esophagus, and lung. However GST-pi levels are not increased in hepatocellular carcinomas, the mouse GST-pi is expressed constitutively in hepatocytes. Therefore, induction of GST-P gene hepatocarcinogenesis is a rat-specific event (Sato, et al, 1989).

Glutathione-S-transferase-P (GST-P) is an enzyme that catalyses the glutathione conjugation of electrophilic xenobiotics. The enzyme is dramatically increased during chemical hepatocarcinogenesis of the rat. This is apparently an induction at the transcriptional level but is somewhat different from usual induction in that it is not reversed by withdrawal of the carcinogen but becomes constitutive in the precancerous liver cells.

The p53 gene was first described in 1979 and was one of the first tumor suppressor genes to be identified. The p53 protein normally functions as transcription factor and is involved in cell cycle regulation, DNA repair, and apoptosis. Abnormalities of the p53 tumor suppressor gene and related pathways are among the most frequent molecular events in human and animal neoplasisa. In more than half of all human tumors,
The mutational inactivation of the p53 gene has been observed (Oren et al. 2002; Vogelstein et al. 2000). The consequence of a non-functional p53 pathway would be loss of protection against harmful compounds and other types of cellular stress. Normal functional p53, on the other hand, protects cells by inducing cell cycle arrest or apoptosis.

Mammalian cell growth and development is regulated by specific patterns of gene expression, which are controlled by promoter elements that can result in transcriptional activation or repression. There are two major epigenetic systems that affect mammalian gene expression at the chromatin level: DNA methylation and histone modification (Bestor et al. 1988; Yen et al. 1992; Okano et al. 1998). Although the role of DNA methylation and histone modifications in gene expression is well established, the primary signals for specific gene expression mediated by these factors may come from cellular stress or DNA damage. In mammalian cells, a functional p53 tumor suppressor protein responds to a variety of cellular stresses, including DNA damage and aberrantly activated oncogenes, and may induce cell cycle arrest and apoptosis (Levine et al. 1997; Vogelstein et al. 2000; Hoh et al. 2002). Approximately 1/10th of human gene promoters contain a p53 binding site and therefore may be classified as p53-responsive genes. Some are transcriptionally activated; others are transcriptionally repressed by p53. The ratio of up-regulated genes to down-regulated genes was equivalent 3:2. Affected genes are involved in the cell cycle, angiogenesis, DNA repair and replication, transcription, and apoptosis.

The induction of an apoptotic response by DNA damage frequently occurs through the activation of the p53 tumor suppressor protein (Vousden et al. 2002). P53 is the most commonly mutated gene in human cancer, a finding that reflects its crucial anti-cancer activity (Levine et al. 1997). P53 acts to obstruct tumorigenesis by serving as a cellular stress and DNA damage sentinel. In response to myriad stresses, including DNA damage, hypoxia, or proliferation 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 of cancer.

The tumor suppressor gene p53 plays an important role in the regulation of the cell cycle. Upon DNA damage, the p53 protein is probably stabilized by post-translational modification, resulting in higher p53 levels (Gottlieb, and Oren, et al 1996). This may lead to blockage of the cell cycle and enhanced DNA repair (Waga et al, 1994; El-deiry et al, 1994; Smith et al, 1994; Marx, et al, 1994; Lane et al, 1992) or it may lead to apoptosis (Yonish-Rouach, et al, 1991; Liebermann, et al, 1995; Saito, and Ogawa, 1995). Mutation of p53, inhibition of p53 functions and complete loss of p53 expression are regarded as important steps in tumor genesis. A mutated p53 is found in >50% of human tumors (Gottlieb, and Oren, et al 1996).

The p53 tumor suppressor protein is a potent inhibitor of cell growth, capable of arresting the cell cycle at several points and, under some circumstances, can participate in DNA repair after genotoxic insult, and can activate the apoptotic machinery leading to cell death (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997; Sharpless and DePinho, 2002; Attardi and Depinho, 2004). The p53 gene is frequently altered in human cancers (Greenblatt et al, 1994). In addition, apoptosis has recently been suggested to be a major contribution to p53 mediated suppression of tumor formation (Attardi and DePinho, 2004). Besides the well-established roles in tumor suppression, a remarkable recent finding has revealed an unexpected developmental role for p53 in that it promotes mesoderm differentiation (Cordenonsi et al, 2003; Takebayashi-Suzuki et al, 2003). Key TGF-β signaling events depend on functional p53, including the full transcriptional activation of the CDK inhibitor p21^{waf1}. The p53 and Smad proteins physically interact and synergistically activate TGF-β-induced target genes (Cordenonsi et al, 2003). Under normal conditions, p53 is a short lived due to degradation upon ubiquitination by Mdm2, a ubiquitin E3 ligase (Michael and Oren, 2003; Vargas et al, 2003). In response to environmental stress, and perhaps developmental cues, it also undergoes a variety of other post translational modifications
including phosphorylation and acetylation (Prives and Manley, 2001; Brooks and Gu, 2003; Xu, 2003). Stress-induced phosphorylation of two critical residues on p53, Ser 15 and Ser 20, results in stabilization and activation of p53 (Chehab et al, 1999; Dumaz and Meek, 1999). In addition to Ser 15 and Ser 20, Ser 46 has been recently shown to be critical for p53 stability regulation (D’Orazi et al, 2002; Hofmann et al, 2002).

Although morphological and biochemical changes are the characteristic features of apoptosis, it involves a complex molecular process. Possible convergence of various events results in the activation of apoptotic signaling pathways. The p53 gene, implicated in animal and human carcinogenesis is a potential regulator of apoptosis. The p53 mutations are now recognized to be the most common genetic changes in human cancers, while apoptosis pathway is to p53 induction, this pathway is held in check by the anti-apoptosis gene bcl-2. The protooncogene bax forms a heterodimer with bcl-2 and initiate apoptosis. Activation of transcription factor NF-kp involving its nuclear translocation has been linked with apoptosis.

The tumor suppressor gene p53, a transcription factor, has been identified as a participant in the cellular DNA damage response. Upon DNA damage, P53 up regulate waf1p21 to cause G1 arrest, which allows time for damaged DNA to be repaired or for triggered apoptosis to eliminate genetically, damaged cells. (Vousden, 2000). The apoptosis promoting capacity of p53 is presumably due to its capability of p53 to activate bax, a gene that encodes an inhibitor of bcl-2. Recent reports indicate that waf1p21 is up regulated by wild type p53 protein; the levels of waf1p21 protein may reflect the functional status of p53.

The present study was designed to evaluate the vitamin D3 on the development of enzyme altered lesions, their positive phenotype for PGST* can demonstrate more numbers and larger sizes of the putative preneoplastic focal lesions than the other markers in various rat liver carcinogens.
**Material 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±10°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 procedure**

Rats were divided into four groups (A-D). 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. Daily food and water intakes were noted and the body weights of the animals from each group were recorded every second day. All the treatments were withdrawn after week 20 and the rats were killed by decapitation between 09:00 and 11:00 h under proper light ether anesthesia after week 21 to carry out experiments. All the animals were fasted over night before being killed.
Fig. 3. Basic experimental protocol for PGST and p53 expression.

- Basal diet and drinking water *ad libitum*;
- Basal diet with 0.05% 2AAF, 5 days a week;
- Basal diet with 0.03μg VD$_3$ / 100μl propylene glycol per OS twice a week;
- Basal diet with 0.05% 2AAF and 0.03μg VD$_3$ / 100μl propylene glycol per OS twice a week;
- Starting treatment;
- Termination of treatment;
- Animals were killed.
Immunohistochemical staining of PGST* expression

The liver slices places into acetone at the death were fixed overnight at -20°C with one change to fresh acetone. (Kaku et al, 1983) sections were placed into poly-L-Lysine-coated slides (0.5% in water; sigma), deparaffinized in xylene and dehydrated with graded alcohol to water. The liver sections were acetone fixed for 5 minutes allowed to dry and then rehydrated in PBS (phosphate buffer saline). Endogenous biotin was blocked by incubation with acidic solution (50 microgram per ml) for 30 min. Non-specific protein binding was minimized by the use of 5% normal serum. The rabbit antirat PGST* antibody was diluted 1:700 in 1% bovine serum albumin (BSA) and incubated with the tissue sections overnight at 4°C. Next, the sections were incubated with a biotinylated secondary antibody (Goat anti-rabbit IgG (Beehringer Mannheim, Germany) diluted 1:200 in 1% BSA) for 30 min at room temperature. This was followed by incubation with streptavidin bound at β-galactose subsequent chromogen development with 5-bromo-4-chloro-3-indoyl β-galactoside (Boehringer Mannheim) was as described by Bondi et al, 1982. The sections were counterstained with mayers hematoxylin and then cover slipped with crystal mount. The area of liver sections were determined with a planimeter and the number of single hepatoocyte expressing PGST* as well as PGST* positive foci were determined per cm² of hepatic tissue. The percentage size distribution in all 2AAF treated rates. PGST* positive lesion composed of more than 11 cells were considered as altered liver cell foci.

Immunohistochemical detection of p53 protein in cold acetone fixed, paraffin embedded in liver sections was performed by the streptavidin avidin-biotin-immunoperoxidase- complex method (Jin et al, 2002), briefly, 5 mm thin sections on poly-L-Lysine coated slides were deparaffinised and dehydrated. Antigen retrieved was facilitated by heating the tissue sections in 10 mm citrate buffer (pH.6) for 15 min in a microwave oven at 750w. There were allowed to stand in hot buffer for an additional 15 min. Endogenous peroxidase activity was blocked with 1% H₂O₂ in 0.1M Tris-NaCl (pH. 7.6) for 30 min. After incubation with 5% normal goat serum for 1 hr at
37°C, sections were incubated overnight at 4°C with the primary antibody sheep anti-p53 antibody (Sigma) at 1:300 dilution in 1% BSA using a 1:50 dilution. Sections were then incubated with a biotinylated secondary antibody goat anti-sheep IgG (Sigma) for 30min at 37°C with 1:200 dilutions. This was followed by incubation with streptavidin preoxidase (1:100) for 1hr and subsequent chromagen development with 0.5% 3,3’-diaminobenzidine tetrahydrochloride (DAB) and 0.33% H2O2 0.5M Tris_ NaCl as the substrate. The section were then counterstained with Harris heamatoxylin, dehydrated and mounted and served as positive control. Negative controls were prepared following all the above-mentioned steps omitting the primary antibody. P53 immunostaining was considered positive when the nuclei of the hepatocytes were stained prominently purplish brown.

**Statistical analysis**

Student’s t-test was performed to compare sample means and the results were expressed as mean ± S.E. Statistical significance was set at P<0.05 for all the values.

**Results**

Table 3 depicts the results of quantitative analysis on the frequency of single PGST*-positive hepatocytes as well as foci. The decrease in total number of single PGST*-positive foci hepatocytes in group D rats were significantly decreased (P< 0.001) when analyzed by student’s t-test. A further analysis of the size distribution of PGST*-positive foci in group B showed that the number of large foci (0.20 sq.mm) which accounted for more than 81.14% of the foci in group B (2-AAF) had fallen to around 51.63% in group D which is supplemented by VD3. However, this decrease in the percentage of large foci was matched by an increase in the numbers of small foci (<0.05 sq.mm).

A few p53 immunopositive cells (0.21±0.06%) was detected in 2AAF control liver sections (fig.3.E), where as (fig.3.F) should increase in p53 immunoreactivity
(1.12±0.29%; P<0.02) upon VD3 supplementation in group D rats when compared to 2AAF control

Table 3: Effect of VD3 on the induction of PGST-positive foci and P53 expression in different groups of rats.

<table>
<thead>
<tr>
<th>group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Single PGST positive cells/cm²</th>
<th>PGST positive foci</th>
<th>Size distribution (%)</th>
<th>P53 leveling index (%)</th>
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<tr>
<td></td>
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<td></td>
<td>No./cm²</td>
<td>Average area (mm²)</td>
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<tr>
<td>C</td>
<td>2-AAF control</td>
<td>10</td>
<td>97.51±10.22</td>
<td>13.26±2.59</td>
<td>8.26±3.94*</td>
<td>7.62</td>
</tr>
<tr>
<td>D</td>
<td>VD3 + 2-AAF</td>
<td>10</td>
<td>42.74±5.19*</td>
<td>7.78±1.78a</td>
<td>2.96±1.92a</td>
<td>36.15</td>
</tr>
</tbody>
</table>

*Values represented mean ± SE (n = 10)

aP<0.001 when compared to 2-AAF control (group-C).
bP<0.02 when compared to 2-AAF control (group-C).
Fig. 3A

**Fig. 3.A.** PGST* positive stained with avidin-biotin-peroxidase (A×100)

Fig. 3B

**Fig. 3.B.** Negative control of PGST* foci stained with avidin-biotin-peroxidase (A×100)
Fig. 3C. PGST* positive stained with avidin-biotin-peroxidase (A×400)

Fig. 3D. Negative control of PGST* foci stained with avidin-biotin-peroxidase (A×400)
Fig. 3E. Light micrographs of tissue sections from rat liver (after 16 weeks) showing immunostaining of p53 (Group C, 2-AAF control) with sheep anti-p53 antibody and DAB; arrow (↑) indicates p53 immunopositive cells. Magnification ×270.

Fig. 3F. Light micrographs of tissue sections from rat liver (after 16 weeks) showing immunostaining of p53 (Group D, VD₃ + 2-AAF) with sheep anti-p53 antibody and DAB; arrow (↑) indicates p53 immunopositive cells. Magnification ×270.
Discussion


Hepatocarcinogens are known to inhibit regeneration of hepatocytes following partial hepatectomy (PH). However hepatocytes in preneoplastic lesions are resistant to the inhibitory effect of carcinogens like 2-acetylaminofluorene (2-AAF) (Solt, et al, 1976; Farber, et al, 1984), responding strongly to proliferative stimuli under conditions which block cell division in the background parenchyma. The functions of GST-P are abundant in the cells of hepatocyte nodules and hepatocellular carcinomas, is unknown, although the fact that there are multifunctional enzymes involved in the detoxification of electrophilic drugs and carcinogens would indicate a crucial role in the mechanism of resistance (Tsuchida, et al, 1992).

The first step in sequential multistep carcinogenesis is initiation and this can be divided into two parts, the biochemical interaction of the carcinogen with cellular components such as DNA (Yan et al, 1998) and the fixation of DNA damage with the formation of a small population of cells histochemically recognized as phenotypically altered preneoplastic lesions. The initiation of carcinogenesis is known to involve the formation of heritable mutational changes in a cell (Pitot, 1986, Pitot, 1989, Pitot et al, 1989). The contribution of cellular proliferation to the initiation process has been well studied, the exact influence of proliferation on the rest of the carcinogenic process, the states of promotion and progression, has not been well established. Several workers have examined cell proliferation in altered hepatic foci (AHF) and have demonstrated an enhanced focal, relative to non focal, proliferation (Rabes et al, 1972; Pugh and Goldfarb, 1978; Barbason and Betz,1981; Schulte-hermann et al,1981) for AHF.
developing under conditions of continuous carcinogen administration, an increase in focal cell proliferation relative to the normal appearing, non focal hepatocytes occurred with carcinogen administration (Rabes and Szymkowiak, 1979). However, Rostein et al (1986) have reported that the focal labeling index did not change with time after AAF administration. These observations imply that several factors can contribute to the observed focal index observed after carcinogen administration, including dose and duration of carcinogen administration as well as the type of lesion induced (Dragon et al, 1994).

In the resistant hepatocyte model (Solt and Farber, 1976), 99-100% of the generated hepatocyte nodules are positive for GST-P during 2-AAF feeding (Tatematsu, et al, 1985). Moore et al, 1987, and Satoh et al, (1989) have proposed that initiated cells may be detected histochemically by virtue of their increased expression of PGST*. In these studies, the number of such single, putative initiated cells varies with time and dose following initiation by 2AAF, rats initiated neonatally with 2AAF, has 1-3x 10⁴ AHF per liver ( Peraino et al; 1984), while in rats given a partial hepatectomy and then initiated with 2AAF as young adults, this number ranged 2.5x5x10⁴ AHF per liver (Xu et al; 1990) This finding was in agreement with our present findings where an increased expression of PGST* positive foci were observed in 2AAF treated rats (Table .3).

A striking observation, however was the dose dependent induction of PGST* in single hepatocytes. These cells accumulate in dense clusters mainly around the central veins, clearly distinguished from the foci. Only a marginal number of PGST* - positive cells were seen in control. PGST* is a valuable marker of preneoplastic foci, nodules and tumours (Sato, 1989) and it is not expressed in normal hepatocytes (Satoh et al., 1985).

The decrease in total number of single PGST* positive foci hepatocytes in group D rats were significantly decreased (P< 0.001) when analyzed by student’s t-test. A further analysis of the size distribution of PGST* positive foci in group B showed that the number of large foci (0.20 sq.mm) which accounted for more than 81.14% of the foci in group B (2-AAF) had fallen to around 51.63% in group D which is supplemented by
VD₃. However, this decrease in the percentage of large foci was matched by an increase in the numbers of small foci (<0.05 sq.mm). It is true that all the cells in the foci are not positive for PGST* and PGST* negative cells are also present which expand into colorless of AHF in presence of promoting agents (Dragon and Pitot, 1992). Previous studies have detected an intrinsically greater labeling index in initiated cells (Schulte-Hermann et al., 1981; Zerban et al., 1989) under the influence of a promoting agent and in the drop in labeling index in AHF with removal of the promoting agent (Ghauert et al., 1986; Dragon and Pitot, 1992). It has been suggested that single hepatocytes that express GST-P are putative initiated cells in rat liver (Moore, et al., 1987; Satoh, et al., 1989; Dragan, et al., 1993) and that the GST-P gene is switched during the initiation process (Moore, et al., 1987; Satoh, et al., 1989). It was recently reported that GST-P expression in putative initiated hepatocytes is controlled by regulators such as the transcription factor AP-1 [c-jun-c-fos complex, positive regulator (Okuda, et al., 1989) and SF-B (silence factor B, negative regulator)] during rat hepatocarcinogenesis (Imagawa et al., 1991).

The p53 tumor suppressor pathway plays a pivotal role in prevention of cancer development and is disabled by mutations or deletions in 50% of all human malignancies (Vogelstein et al., 2000; Hall, et al, 1997; Hainaut, et al., 2000). The p53 is a potent transcription factor that can activate a subset of genes controlling the progression of the cell cycle and the activation of proapoptotic signals (Levine, et al, 1991; Kastab, et al, 1995). In normal proliferating cells, p53 is kept at a very low level by MDM2, which inhibits p53 transcriptional activity and facilitates its ubiquitin-dependent degradation (Freedman, et al, 1999). However the p53-MDM2 regulatory loop can be disrupted in response to stress signals. This leads to p53 protein stabilization, nuclear accumulation and activation of the p53 pathway, resulting in growth arrest or apoptosis (Momand, et al, 2000).

Loss of control of genomic stability is central to the development of neoplasm and p53, by regulating normal responses to DNA damage and other forms of genotoxic stress, is a key element in maintaining genomic stability. Thus it is not surprising that functional p53 is lost in about half of all human cancers. What about the other half? The
possibility is that, p53 independent regulatory mechanisms have been lost. Another is that inactivation of p53 dependent pathways can occur at any of several different points and that p53 itself is merely the most common target. For example, the p53 inhibitor MDM2 is over expressed in tumors independently of the p53 mutation. It is clear that, p53 is the central component of a complex network of signaling pathways and that the other components of these pathways pose alternative targets for inactivation.

The amount of p53 protein increases in response to variety of signals, such as damaged DNA, arrest of DNA, or RNA synthesis, or nucleotide depletion. The ubiquitin pathway probably plays an important role in degrading p53 (Maki and Howley, 1997) and evidence for a ubiquitin-independent mechanism of degradation has also been presented. How does p53 sense signals? Several known proteins are suspects, the DNA dependent protein kinase (DNAPK), a plausible candidate, binds to and is activated by broken ends of DNA and can phosphorylate residues at 15 and 37 of p53 in a DNA-dependent manner in vitro (Lees-Miller et al, 1992). The Phosphorylation of serine 15 affects the transactivation and growth arrest function of p53 in some cells. However, cells lacking CNAPH show no defect in the p53-mediated inhibition of the cell cycle, revealing that if DNAPK has any role in regulating p53 at all, other components must be able to compensate for its loss. Many protein kinases have been shown to phosphorylate p53 in vitro and are candidates for upstream regulators (Ko and Prives, 1996). However, very little in vivo evidence exists for the role of phosphorylation in regulating p53. Recent work shows that p53 can be acetylated in vitro is intriguing and suggests the possibility of an additional mechanism of regulation (Gu and Roeder, 1997).

Recently a role for oncogenic rats and the mitogen-activated protein (MAP) kinase pathway in p53 modulation and function have been revealed in both human and rodent cells. High expression of rats or activation of the MOS/MAPK pathway induces wild-type p53 levels and causes a permanent growth arrest, similar to cellular senescence (Fukasawa and Vande woude, 1997). Cells lacking p53 can tolerate high levels of MAPK and display loss of p53 dependent cell cycle arrest and enhanced genomic stability. In a cell line defective in the MAPKinase pathway and in p53 expression,
increased expression, increased expression of the MAP kinase ERK2 restores the normal levels of p53, clearly placing ERK2 in a pathway that regulates the steady state level of p53. MAPK has been shown to phosphorylate residue 73 or 83 of murine p53 in vitro, and this phosphorylation may be important in stabilizing the protein (Milne et al, 1997). Other kinases, such as DNAPK II, cyclin A-Adc2, and Cyclin B-Cdc2 are known to phosphorylate the p53 protein invivo and may play a role in stabilizing it.

P53 plays a role in triggering apoptosis in certain cell types. Stimuli such as DNA damage, withdrawal of growth factors, and expression of myc or E1A can also cause P53 dependent apoptosis (Clarke et al, 1993). P53 must be able to function as a transcription factor to block the G1-S transition, but p53 mediated apoptosis does not necessarily require transcriptional activation, because inhibition of transcription by actinomycin D or translation by cycloheximide does not always affect p53-dependent apoptosis in the absence of transactivation (Yan et al, 1998). However, the pro-apoptotic proteins Bax and IgFBP-3 are transcriptional targets of p53, suggesting that transactivation by p53 is important in inducing apoptosis in some circumstances. In addition, the anti-apoptotic proteins Bcl2 and the adenovirus 19-KDa E1B protein can prevent p53-mediated apoptosis.


Loss of p53 protein function appears to be an important step in tumor development, because mutations in, or allelic loss of the p53 gene have been found in 50% of the human tumors in a wide variety of organs including colon, lung, bladder, ovary, and skin (Carson, et al, 1995). Loss of p53 may have important consequences for the cell although p53 does not appear to be essential for cell cycle regulation under normal
developmental conditions: mice lacking the p53 gene develop and grow normally, although they are tumor-prone (Donehower, et al, 1992; Kemp, et al, 1995). An important physiological function of p53, however, seems to be regulating the cell cycle after induction of DNA damage by blocking the cell cycle in G1-phase. This allows the cell to repair its DNA-damage, or may trigger apoptosis (Yonish-Rouach, et al, 1991; Liebermann, et al, 1995; Saito, and Ogawa, et al, 1995). Therefore, p53 has been regarded as a guardian of the genome (Lane, et al, 1992).

Our data reveals that the unique protective role of VD₃ against chemically induced liver tumorigenesis in rats. Regardless of the mechanism, based on the results reported here, VD₃ triggers a unique protective effect against the expression of PGST* positive foci and a significant up regulation of p53 protein expression in chemically induced hepatocarcinogenesis in rat. This attribute could be considered important as dietary antioxidant may open new perspective for the human liver preneoplasia in near future and Long-term supplementation of VD₃, there by indicating the potential modulatory role of VD₃ against neoplastic transformation.