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MALIGNANT TRANSFORMATION OF THE HUMAN FIBROBLAST CELL STRAIN MSU-1.1 BY ULTRAVIOLET RADIATION: CORRELATION OF TUMORIGENICITY WITH ANCHORAGE INDEPENDENCE AND GROWTH FACTOR INDEPENDENCE

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ABSTRACT

MALIGNANT TRANSFORMATION OF HUMAN FIBROBLAST CELL STRAIN MSU-1.1 BY ULTRAVIOLET RADIATION: CORRELATION OF TUMORIGENICITY WITH ANCHORAGE INDEPENDENCE AND GROWTH FACTOR INDEPENDENCE

By

Sardar Waheed Ashraf-khan

I irradiated MSU-1.1 cells, an immortal, non-tumorigenic human fibroblast cell strain with a stable, near diploid karyotype with 254 nm UV radiation and selected for focus formation. A dose-dependent increase in focus formation was observed. From each of the eight independent irradiated cell populations, 5-6 focus-derived cell strains were selected and tested for tumorigenicity by injecting them into athymic mice. When a cell strain from an irradiated cell population made a tumor, this cell strain and a focus-derived cell strain not able to form a tumor from the same population were selected for further analysis. Five UV-induced focus-derived cell strains formed high grade spindle cell sarcomas in athymic mice at a high frequency and with a very short latency.

These five cell strains grew well in agarose and in medium without exogenous growth factors. Two UV-induced focus-derived cell strains and one control focus-derived cell strain also formed fibrosarcomas in athymic mice but at a lower frequency and with a longer latency. These three cell strains did not grow well in agarose or in medium without exogenous growth factors. However, two cell lines derived from the tumors (fibrosarcomas) grew well in agarose and in medium without exogenous growth factors. One UV-induced focus-derived cell strain formed a single fibroma after a long latency. The cells that formed the fibroma and the fibroma-derived cell line did not grow in agarose or in medium without exogenous growth factors. All the non-tumorigenic UV-induced focus-derived cell strains did not grow in agarose and in medium without exogenous growth factors. In summary, UV radiation can transform MSU-1.1 cells to focus formation in a dose-dependent manner and some of these focus-derived cell strains can form tumors in athymic mice. Growth in agarose and in medium without exogenous growth factors are two reliable in vitro characteristics which correlate strongly with tumorigenicity.

DEDICATED:

To my late father,

whose life and whose death due to cancer inspired me,

To my wife,

whose love, support and patience helped me,

To my children,

whose proper education motivated me,

and to millions of cancer patients,

whose untold suffering and miseries touched and moved me.

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LITERATURE REVIEW

A. MULTI-STEP MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

Human cell transformation results from a multi-step process. Multiple genetic changes in specific proto-oncogenes and/or tumor suppressor genes are required to transform a normal cell into a malignant cell. In this review, I will be using the terms "transformation", "neoplastic transformation" and "malignant transformation" as defined by the Tissue Culture Association (Schaffer, 1983). The term "transformation" indicates that the cells described have one or more of the phenotypic characteristics of tumor cell(s), e.g., morphological alteration, growth factor independence, anchorage independence, increased saturation density, lack of contact inhibition. The term "neoplastic transformation" means that the cells described form benign or malignant tumors when they are injected into a susceptible animal host like athymic mice. "Malignant transformation" means that cells form a malignant tumor when they are injected into a susceptible animal host.

B. CHEMICAL CARCINOGENESIS

The first report showing an association between cancer and chemical exposure was in 1761 by the English physician, John Hill. He noted a higher incidence of oral and nasal cancer in snuff users (Hill,1761). In 1775, Sir Percival Pott, a surgeon at St. Bartholomew Hospital in London, described the possible relationship between exposure to chimney soot and scrotal cancer (Pott, 1775). His work provided the first evidence that environmental factors could play a role in causing human cancer. In 1795, Samuel von Soemmerring reported that there was an association between lip cancer and clay pipe smoking (Soemmerring, 1795).

In the next 90 years, little was published indicating an association between exposure to chemicals and the induction of cancer. However, in 1879, Harting and Hesse showed that occupational exposure to benzene, an important industrial chemical is carcinogenic. This was followed by a report by Rehn who pointed out that the incidence of bladder cancer was higher in workers who were exposed to aromatic amines (Rehn, 1895). These reports led to the first experimental induction of tumors in animals by the two Japanese pathologists, Yamagiwa and Ichikawa. They applied coal tar, a complex

mixture of chemicals, to the ears of rabbits. Tumors developed at the site of application (Yamagiwa and Ichikawa, 1918). Sir Earnest Kennaway was the first to demonstrate that a specific chemical, dibenzanthracene, was carcinogenic in experimental animals (Kennaway and Hieger, 1930).

The first evidence linking hormones to cancer was provided by Lacassagne in 1932. He experimentally induced mammary tumors in mice by treating them with estrone. Three years later, it was found that the rats fed with an azo-dye, o-aminoazotoulene developed liver tumors (Sasaki and Yoshida, 1935). Hueper et al. (1938) made similar findings with 2-naphthylamine. In the period 1950 to 1967, several workers showed that naturally occurring chemicals like alkaloids from plants, e.g., braken fern (Pteridium aquilinum) (Evans and Mason,1965), cycads (Laqueuer, 1964) and Scenecio (Cook et al., 1950) and some toxins from fungi e.g., aflatoxin B1 from Asperigellus (Wogan,1966; Wogan and Newberne, 1967) are carcinogenic in experimental animals.

The association of dietary factors with human cancer was first shown by Alpert and colleagues in 1968. These workers found that the incidence of hepatocellular carcinoma in Africa was correlated with exposure to high levels of aflatoxin in

diet (Alpert et al., 1968). In the same year, animal bioassays were developed as a method to identify chemical carcinogens (van Daurren et al., 1968). In the last 20 years, epidemiological studies (e.g.,Doll & Peto, 1981), laboratory studies (e.g.,McCormick and Maher, 1989), and clinical studies(e.g., Huff, 1992) have provided evidence that there is an association between exposure to certain chemicals and cancer.

1.0 MECHANISM BY WHICH SOME CHEMICAL AGENTS PLAY A ROLE IN CAUSING CANCER

Classically, chemical carcinogenesis has been described on the basis of a phenomena now widely known as "initiation-promotion". The pioneer experiments demonstrating this phenomenon were performed by Rous and Kidd (1941) on the skin of rabbit and by Brenblum and Shubik (1947) on the skin of mice. In these experiments, a single dose of 7,12-dimethylbenz(a)anthracene (DMBA), a carcinogenic polycyclic aromatic hydrocarbon (PAH), was applied to the animals' skin at such a low concentration that no skin tumors were formed during the lifetime of the animals. Croton oil was referred to as a "promoter" because the compound does not cause tumors when applied to the skin but allows a non-carcinogenic dose of

another chemical to produce tumors. However when animals were treated with DMBA as described followed by regular application of croton oil, usually twice a week, multiple benign tumors (papillomas) arose. A long delay in beginning of the application of the promoter, up to even up to a year, led to as many tumors as if croton oil application was begun a week or two after the DMBA. Papillomas, so formed, almost always regressed when one ceased application of the promoter. Subsequent application(s) of DMBA or similar agents caused some of the benign tumors to progress to malignancy, suggesting that additional genetic changes were required for malignant cellular transformation (Hennings et al., 1983; Balmain and Brown, 1988; Balmain et al., 1991). In this procedure, the treatment with the low dose of carcinogen (DMBA) is referred to as "initiation", the treatment with croton oil as "promotion"

It is now widely accepted that initiation involves an irreversible, rapid and permanent change in the cell genome and that promotion involves some modification of gene expression and clonal expansion of the initiated cells (Newbold and Overell,1983; Vogelstein et al.,1988; Agarwal and Mukhtar, 1992). Although the initiation- promotion theory

has been widely accepted, it cannot be considered to be an adequate description of chemical carcinogenesis. For example, the theory implies that the promoters themselves are non-carcinogenic and their action is mainly epigenetic. However, there are reports indicating that TPA, the active ingredient of croton oil, a commonly used promoter, can cause chromosomal damage (Iversen,1986). Even more important, sometimes tumors appear in a dose-dependent fashion after TPA treatment alone (Iversen, 1986). In fact, the initiation-promotion theory is basically a description of a commonly seen phenomenon and does not attempt to describe the precise underlying molecular and cellular events that occur in the process of chemical neoplastic transformation (Weisburger and Williams, 1982; Bremmer et al, 1994).

In the last 20 years, through the use of molecular biochemistry, the molecular and cellular events responsible for the malignant transformation of animal and human cells are becoming more clear (Burns and Balmain, 1992; Burns et al., 1991). It is now widely accepted that malignant transformation in vivo as well as in vitro involves multiple sequential genetic and possibly epigenetic changes. Each change causes the cell to be one step closer to the final

overt stage of neoplastic transformation (Bishop, 1987, 1991; McCormick & Maher, 1994). In tumors of adult humans and in the most recent in-vitro cellular transformation models, the number of independent genetic events required to change a normal cell to malignant may be six or more (Fearon & Vogelstein, 1990; McCormick and Maher, 1994). The discovery of proto-oncogenes and tumor suppressor genes, which are now considered to be the molecular targets of chemical and physical carcinogens (Barbacid, 1987; Balmain and Pragnell 1983; Weinberg, 1989), the knowledge of endogenous mutagenic mechanisms such as DNA polymerase infidelity (Wang et al al., 1991), oxygen radical DNA damage (Cerutti, 1985; Autrup and Harris, 1983; Perchellet et al., 1994), and the role of carcinogenic agents as mutagens (Maher et al., 1968; Ames and Gold, 1990; Yuspa and Harris, 1982) necessitate that the classical views of initiation-promotion be revisited.

C. VIRAL CARCINOGENESIS

In addition to the evidence that certain chemicals can cause cancer (Harris, 1991), there is ample evidence that some viruses can play a causal role in causing specific types of human and animal cancers (Evans and Mueller, 1990; Masucci, 1993; Chow, 1993). In the case of all such viruses, many

individuals are infected with such viruses during their life time, but only a few develop viral-associated cancers (Mayer and Ebbsen, 1994). This suggests that additional genetic and/or possibly some epigenetic changes are required for malignant transformation (Vogelstein and Kinzler, 1992a). Viruses are classified according to the type of nucleic acid in their genome. In the following pages, the role of both DNA and RNA viruses in carcinogenesis will be discussed.

1. DNA VIRUSES AND CANCER

DNA viruses normally infect a cell and complete their life cycle by replication in a permissive host resulting in the lysis of the cell and the release of newly synthesized viruses. In a non-permissive host cell, integrated viral DNA cannot complete its replication cycle because during the process of integration, the viral genes essential for the completion of replication are interrupted or lost (Kleinheinz et al., 1989). The early viral genes, those transcribed early in the synthesis of the viral genome, are important for cellular transformation. When the early viral genes are expressed continuously, this sometimes results in a disruption of the normal growth control of the infected cell leading to

cellular transformation (Segawa et al., 1993). Some of the important DNA viruses (papova viruses, adenovirus and herpes virus) and their transforming protein(s) are discussed below.

2.0. Papova Viruses

The Papova family of viruses can be divided into two main groups on the basis of their structure and functions i.e., the papilloma viruses and the polyoma viruses.

2.1. Papilloma Viruses and Cancer:

Both experimental studies (Khan et al., 1993) and epidemiological studies (Schiffman et al., 1993; Isacsohn et al., 1994) provide strong evidence that human papilloma viral infection is associated with human cervical cancer (Morrison et al., 1994; Khan, 1993), oral, and certain other anogenital malignancies (zur Hausen, 1994a; Lu-Lg, 1993; Munoz et al., 1993). Detection of low levels of human HPV DNA sequences in cancer cell lines derived from two well-differentiated nasopharyngeal carcinoma suggest that they may have had a role in the development of these tumors also (Huang et al., 1993; Lowhagen et al., 1993).

The papilloma viruses can be divided into high and low risk viruses. High risk viruses like HPV 16 and 18 are strongly associated with invasive squamous cell carcinoma of

the cervix (Lu-Lg, 1993). According to one study, the DNA of these high risk HPV types is detectable in 90% of cervical carcinoma biopsies (zur Hausen, 1994a). In contrast, the low risk HPV viruses like HPV type 6 and HPV type 11, are associated with benign lesions such as condyloma acuminata (Laimins, 1993).

Molecular studies of HPV associated cancers have revealed that the integration of viral DNA into the host cell results in the interruption of the E1/E2 open reading frames (ORF) of the viral genome (zur Hausen, 1994b). Since the E2 region of the viral genome represses the transcription of early viral proteins E6 and E7, a disruption in the E2 gene results in an over expression of E6 and E7 proteins (seo-Ys et al., 1993). E6 and E7 viral proteins are thought to play an important role in the etiopathogenesis of certain anogenital cancers (Yamashita et al., 1993; zur Hausen, 1994b).

In-vitro studies have also provided evidence of role of HPV in cellular transformation (Wantanabe et al., 1989; Wazer et al., 1995). Both the *E6* and *E7* genes of the high risk HPV demonstrate the ability to transform human and rodent cells in vitro (Band et al.,1991; Howley,1991). E6 and E7 proteins can render some cells immortal and can also confer on the cells

the ability to grow in soft agar (Reznikoff et al., 1993). Some of these transformed cells can form tumors when injected into athymic mice (Munger and Phelps et al., 1993; Howley, 1991; Dipaolo et al., 1993). Von-Knebbel et al. (1988) provided additional evidence that E6 and E7 proteins play a causal role in the development of cancer. They showed that expression of HPV 18 *E6-E7* antisense RNA resulted in the inhibition of tumorigenicity in athymic mice of C4-1 cervical cancer cell line.

In summary, in-vitro studies strongly suggest that HPV plays an important role in cellular transformation of some specific human and animal cells.

2.1.1 REGULATION OF HUMAN PAPILLOMA VIRUS ONCOPROTEINS

HPV onco-proteins are transcriptionally regulated by both viral encoded factors and certain other cellular factors (Cripe et al., 1990; Cuthill et al., 1993; Khan et al., 1993a). Papilloma virus oncoproteins are regulated by the E2 viral protein which acts as a transactivator or in certain cases as an encoded repressor (Seo-Ys et al., 1993a). In addition, a wide variety of cellular proteins have been reported to influence the transcription of E6 and E7 proteins such as activator protein-1(AP-1) (Chong et al., 1990; Offord

et al., 1993) and nuclear factor-1(NF-1) (Gloss et al., 1989; Apte et al., 1993). A detailed review of the transcriptional regulation of HPV is given by Hoppe-Seyler and Butz, (1994). Recently, some workers have shown that HPV transcription can be suppressed by methylation of the regulatory region, thus preventing the binding of methylation sensitive papilloma virus transcriptional factors (List et al., 1994).

2.1.2. MOLECULAR MECHANISM(S) OF HPV ONCOPROTEIN REGULATION

Molecular studies have given certain clues as to how HPV oncoproteins are regulated by different viral and cellular factors. (Bernard and Apte, 1994; Seo-Ys et al., 1993b). The activation of E6 or E7 transcription could either be due to an increased activity of the transcriptional stimulator or due to functional inactivation of the repressor of transcription (Ibaraki et al., 1993). The resulting over-expression of HPV oncoproteins could directly or indirectly increase the transcription of some other growth related genes, leading to aberrant cellular growth (Munger and Phelps, 1993; zur Hausen, 1994a). The different cellular and viral factors which regulate the transcriptional activity of early gene products may themselves be under the control of other regulatory

proteins (Imler et al., 1988). For example, recently it has been shown that the c-H-ras protein stimulates AP-1 binding to the binding sequences in the upstream regulatory region and results in enhanced transcription of the early viral proteins E6/E7 (Pinion et al., 1991). Further molecular studies aimed at the factors regulating the transcription of the HPV oncoproteins and identifying the biochemical substrates of these viral oncoproteins need to be done. These will help in understanding the role of these factors in the growth regulatory pathways involved in cellular transformation.

2.1.3. HPV ONCOPROTEINS AND TUMOR SUPPRESSOR PROTEINS

A novel mechanism by which HPV E6/E7 proteins play their role in malignant cellular transformation is by interacting with some of the tumor suppressor gene products (Peacock and Bernchimol, 1994; Peacock et al.,1995). E6 proteins of high risk HPV type 16 and 18 can associate with tumor suppressor protein p53 (Vousden et al.,1993; Band et al., 1990). This results in either degradation (Band et al., 1993) or functional inactivation of the p53 protein (Vousden, 1993). Recently, it has been shown that this binding of E6 protein with the p53 protein results in ubiquitin-mediated metabolic processes which result in degradation of the p53

protein (Schaffener et al., 1993). E6-p53 complex formation might also lead to abrogation of p53-mediated DNA damage repair. Kastan and colleagues showed that p53-dependent G1 arrest involves pRb-related proteins and this G1 arrest is disrupted by HPV type 16 E7 protein(Slebos et al., 1994). This would be expected to result in an enhanced rate of mutation formation which could account for the high frequency of cervical and other HPV related anogenital malignancies (Guz et al., 1994). Hence the expression of E6 oncoproteins in certain cells may have the same functional consequences as are seen when the *p53* gene itself is mutated (Butz and Hoppe-Seyyler, 1993; Band et al, 1991).

HPV E7 oncoprotein can form a complex with the Rb protein (Hunag et al.,1993; Dyson et al., 1989). Recently, von-Knebel et al. (1994) suggested that E7's association with Rb results in the release of transcription factor E2F from its complex with pRb. This E7-induced increase in the unbound form of E2F might contribute to the transformation potential of the viral oncoproteins (von-Knebbel et al., 1994). The E7-Rb complex also causes the functional inactivation of the Rb protein resulting in enhanced cellular growth (Dyson et al., 1989). In summary, HPV oncoprotein interaction with tumor suppressor

proteins alone, or in concert with some other genetic change(s) may result in deregulated cell growth, which could contribute to the malignant transformation of a cell (Hope-Seyler and Butz, 1994; Shay et al., 1993).

In spite of these recent discoveries explaining the interaction between p53 and Rb and the HPV oncoproteins (Hoppe-Seyler and Butz, 1994), additional studies are needed to understand some other very important aspects of HPV oncogenesis. Some of the important areas in this respect are: the role of immunological response to HPV in HPV induced cancers (Sandvej et al., 1993), the interaction of HPV with the proteins of other viruses such as those of Herpes group (Evans and Mueller,1990) and the human immunodeficiency virus (Vernon et al., 1992), the cooperation of HPV oncogenes with proto-oncogenes (von-Knebbel, 1992; Masucci, 1993; Jackson et al., 1993), the different intracellular signaling pathways involved in regulation of HPV (zur-Hausen, 1994a), and their precise role in a multi-step schema of carcinogenesis (Dipaolo et al., 1993). Although HPV can play a causal role in immortalization and transformation of specific types of human and rodent cells, additional genetic and/or epigenetic change(s) are required for malignant transformation (Brown et

al., 1994).

2.2. POLYOMA VIRUS AND CANCER

Polyoma virus can transform human and rodent cells in culture (Mess and Hassel, 1982; Jelinek et al., 1992). Some of these polyoma transformed cells form tumors in mice (Dilworth, 1990). The genome of the polyoma virus is a circular double stranded DNA and consists of early and late regions. The early region encodes for three regulatory proteins called large T, middle T and small t antigens. The late region encodes for the viral structural proteins(Friedmann et al., 1979; Evans and Mueller, 1990).

Polyoma small t antigen acts as an auxiliary protein that increases the efficiency of transformation by the other polyoma T antigens. It is dispensable for cellular transformation at least under laboratory conditions. Polyoma large T antigen is a nuclear protein that binds specifically to DNA (Hunter et al., 1978). It has an ATPase activity and it is absolutely required for replication of viral DNA. The expression of polyoma large T antigen in some cells can result in immortalization of the cells (Freund et al., 1992).

However, cells expressing large T antigen are not tumorigenic, indicating the need of additional genetic changes for malignant transformation (Freund et al., 1992; Galiana et al.,1995).

The transformation function of polyoma virus is performed by the middle T antigen, which is an extranuclear protein. Middle T antigen can transform established (immortalized) cell lines but alone cannot immortalize cells in culture (Chastre et al., 1993). Since the middle T antigen is the most important of the three viral proteins, it will be discussed in some detail.

2.2.1 MECHANISMS BY WHICH MIDDLE T ANTIGEN PLAYS A ROLE IN CANCER

In 1984, Bolen et al. detected a tyrosine specific protein kinase activity in the immunoprecipitates of the middle T antigen (Bolen et al., 1984). However, the polyoma middle T antigen does not have any intrinsic kinase activity. This dilemma was solved when it was found that the middle T antigen forms a complex with the cellular homologue of pp60 v-src called pp60 c-src, (Courtneidge & Hebner, 1987) and that pp60 c-src is responsible for the tyrosine kinase activity of the complex. The importance of this complex in viral-mediated

cellular transformation was demonstrated in subsequent studies (Delage et al.,1993; Courtneidge & Hebner, 1987). The pp60 c-src binding site was present in the N-terminal half of the middle T protein. This interaction results in markedly increased pp60 c-src tyrosine kinase activity (Bolen et al., 1984). The increased kinase activity is due to the fact that the middle T antigen locks the src enzyme into a configuration in which it cannot be inactivated by phosphorylation (Courtneidge et al., 1993). These studies led to the hypothesis that by binding to pp60 c-src, middle T antigen activates the oncogene product of pp60 c-src, enabling it to deregulate cellular growth resulting in transformation.

Besides the pp60 c-src, middle T antigen has been reported to bind with other src-related tyrosine kinases such as c-fyn, c-yes and c-fgr (Chang et al.,1990; Kaplan et al., 1989). Thomas et al. (1993) examined the ability of middle T oncogene to transform cells that lack a functional src gene. These workers showed that residual levels of other src related tyrosine kinase such as yes, fyn and phosphotidyl inositol kinase activity appeared to be responsible for cellular transformation and tumor induction in absence of src (Thomas et al., 1993).

Giaever et al. (1993) characterized the phosphoinositide metabolism in a polyoma virus transformed pancreatic islet cell line. They showed that in these cells, phosphoinositide hydrolysis is constitutively activated at the level of phospholipase C resulting in the loss of cellular regulatory control (Giaever et al.,1993). Other workers have demonstrated oncogene cooperation between polyoma middle T oncogene and other cellular oncogenes in polyoma mediated cellular transformation (Hunter, 1978). For example, Chastre et al. (1993) has shown that transfer of the ras and polyoma middle T oncogene to human and rat intestinal cell lines results in neoplastic transformation. Talmage and Listerud (1994) identified the c-fos proto-oncogene as a key nuclear target for middle T-dependent transformation.

In spite of the developments in understanding the pp60 middle T antigen interaction, much still needs to be known to understand molecular events which ultimately result in the increased DNA synthesis and aberrant cellular growth (Hunter et al. , 1991 ; Guy et al., 1992; Wang et al., 1994; Krauzewicz, 1994). One such study to find the downstream effectors in polyoma virus mediated cell transformation was done by Raptis et al. (1990). These workers investigated the

role of cellular ras in the neoplastic transformation of polyoma virus. Murine C3H10T1/2 fibroblasts were rendered ras deficient by transfection with an antisense ras gene construct. Ras deficiency resulted in a partial suppression of the polyoma virus induced transformation. They further showed that the association of polyoma middle T antigen and pp60 c-src in these ras deficient cells led to an increase in protein tyrosine activity. Some of the phenotypic indicators of cellular transformation like focus formation on a confluent monolayer of cells and ability to grow in soft agar were greatly reduced in the cells containing pp60-middle T antigen complexes with reduced p21 ras levels. In the light of these results these workers suggested that ras proteins are needed for the full transformation of C3H10T1/2 mouse cells by polyoma virus. When middle T antigen interacts with the pp60 c-src, the protein itself is phosphorylated on specific tyrosine residues. Some of these phosphorylated residues act as binding sites for the SH2 domains of cellular transforming protein SHC (Van der Geer et al., 1995; Dilworth et al., 1994). Campbell et al. (1994) showed that polyoma middle T antigen interacts with SHC protein through the NPTY (Asn-Pro-Thr-Tyr) motifs. SHC protein on binding to the

phosphorylated tyrosine residue 250 itself also gets tyrosine phosphorylated and binds to the SH2 domain of Grb2 (Campbell et al., 1994). This in turn stimulates p21 ras activity through the mammalian homologue of the Dorsopholia nucleotide exchange factor viz., SOS (McCormick, 1994). Dilworth et al. (1994), As a result of the middle T antigen binding and tyrosine phosphorylation, Dilworth et al.(1994) suggested that middle T antigen-pp60 c-src-SHC-Grb2-sos-ras signaling pathway indicates that middle T antigen should be considered a functional homologue of an activated tyrosine kinase associated growth factor receptor.

In summary, in-vitro and in-vivo studies have helped us to understand many of the molecular and biochemical events involved in polyoma mediated cellular transformation. However, detailed work still needs to be done to understand the precise role of polyoma middle T protein in the multi-step malignant transformation of cells.

3.0. SIMIAN VIRUS 40 (SV40) AND CANCER

In-vitro and In-vivo studies indicate that SV-40 plays an important role in cellular transformation (Reinhart et al., 1993). SV40 is a circular double stranded DNA with 5243 base pairs. The viral genome is divided into two functionally

distinct regions of almost equal size: the early region and late region. The early region codes for two important regulatory proteins i.e., large T antigen and small t antigen. The late viral region codes for the structural proteins of the virus coat (Dilworth, 1990). SV40 is a monkey virus, but there are no reports that SV40 is causative of cancer in monkeys. SV40 transformed rodent cells are usually malignantly transformed, human cells are not. The reason for this difference is not known. However the ability of the virus to immortalize cells in culture even if not malignantly transformed makes it useful (Evans and Mueller, 1990). In-vitro cellular transformation studies involving SV40 tumor antigens may help us to understand some of the events that play an important role in human and animal cancer (Reddel et al., 1995).

Several reports are available which show that SV40 large T antigen can result in immortalization of human cells (Lechner and Laiminis, 1991; Hoffman et al., 1992). For example, Reznikoff and colleagues transfected SV40 large T antigen into uroepithelial cells and obtained SV40 large T antigen expressing cells. After subsequent selection, a few of SV40 large T antigen expressing uroepithelial cell became

immortal (Kao et al., 1993). In addition to the cell culture studies, the development of transgenic mice harboring SV40 large T antigen has enabled us to better understand the role of this oncoprotein in malignant transformation (Hino et al., 1991; Kitagawa et al., 1991). Since SV40 large T antigen is the major viral protein implicated in the cellular transformation, it will be discussed in some detail.

3.1. SIMIAN VIRUS LARGE T ANTIGEN IN CELLULAR TRANSFORMATION

SV40 large T antigen plays an important role in cellular transformation (for review, see Livingston & Bradley, 1987; Dilworth, 1990). SV40 large T antigen is a DNA binding protein which functions as a transcription factor and also plays a role in DNA replication (Schirmbeck and Deppert,1991; Murakami and Hurwitz, 1993; Amin et al., 1994; Boyer et al., 1993; Stahl and Knipper, 1983). The large T antigen is a 708 amino acid phosphoprotein with distinct functional domains. Evidence suggests that the amino terminal-130 residues of T antigen may be sufficient for its transformation ability (Fanning et al., 1984)

3.2. MOLECULAR MECHANISMS OF SV40 LARGE T ANTIGEN MEDIATED CELLULAR TRANSFORMATION

There are several different ways by which the expression

of large T antigen may result in cellular transformation: interaction with signal transduction pathways, interaction with tumor suppressor proteins, and release of certain transcriptional factors and cell cycle regulatory proteins.

3.2.1. SV40 Large T Antigen and Signal Transduction

SV40 large T antigen might play a role in cellular transformation by interacting with the different components of the signal transduction pathway. Some researchers have shown epidermal growth factor and platelet derived growth factors are essential components of the intricate pathway of SV40 mediated cellular transformation (Masuda et al., 1992). Valentinis et al. (1994) studied the role of insulin like growth factor (IGF) receptor in the transformation of Balb/C 3T3 cells by SV40 large T antigen, using a temperature sensitive mutant of SV40 large T antigen. By comparing cells over-expressing the IGF-1 receptor and cells expressing an IGF-1 gene with targeted disruption, they showed that the activation of IGF-1 by its ligand plays an important role in the ability of SV40 large T antigen to promote growth under low serum conditions (Porcu et al., 1992). Sell et al. (1993) confirmed this study by showing that SV40 large T antigen

cannot transform mouse embryonic fibroblasts lacking IGF-1 receptors. These and several other recently conducted studies indicate that signaling via the IGF-1 receptor plays an important role in SV40 large T antigen regulated cell transformation (Porcu et al., 1992; Valentinis et al., 1994).

3.2.2. SV40 Large T Antigen and Tumor Suppressor Genes

SV40 Large T antigen can also lead to aberrant cellular growth by complexing with the p53 tumor suppressor protein (Lamb and Crawford, 1986; Sun et al., 1993) resulting in metabolic stabilization and functional inactivation of p53 protein (Deppert and Steinmayer, 1989; Deppert, 1994). The T antigen-p53 complex cannot bind to the p53 DNA binding sequences resulting in abolition of p53 transactivation. This leads to release of the cell from p53 mediated growth inhibition (Ludlow,1993). Another way by which SV40 large T antigens can inactivate p53 is by inducing a protein kinase which is responsible for the phosphorylation of the cellular p53 protein resulting in its inactivation (Muller et al., 1993; Muller and Scheidtmann, 1995). SV40-mediated inactivation of the p53 tumor suppressor protein, either directly by complex formation or indirectly by metabolic

stabilization of p53 (Deppert et al., 1989), might be an important step in immortalization and/or aberrant cellular growth (Ludlow et al., 1990).

Besides p53, SV40 large T antigen can associate with the underphosphorylated form of the Rb protein (DeCaprio et al., 1988; Jones et al., 1992b). This abolishes the growth suppressor activity of Rb. Recently some researchers have shown interaction of T antigen with some other proteins like p107 which is structurally and functionally closely related to the Rb protein (Amin et al., 1994). This interaction might also be important in SV40 mediated cellular transformation. In summary, circumstantial evidence supports the hypothesis that the interaction of SV40 large T antigen with tumor suppressor gene products plays an important role in mitigating the negative cellular growth control, resulting in viral-mediated transformation.

3.2.3. SV40 and Transcription Factors/Cell Cycle Regulatory Proteins

SV40 large T antigen, by forming complexes with tumor suppressor proteins, might result in the release of transcription factors like E2F (Cao et al., 1992). E2F release might cause an increased transcription of some of the

growth promoting genes resulting in cellular transformation. In the uninfected cells the Rb protein and the closely related protein, p107, interact with cellular transcriptional factor E2F and/or DRTF1, another related protein (Dyson et al., 1993). The Rb-E2F complex dissociates near the G1-S boundary before the initiation of the S phase of the cell cycle. This allows E2F to activate the transcription of certain downstream genes. In the case of SV40 viral infection, SV40 large T antigen associates with the Rb protein and this allows the constitutive release of E2F. Recently, some researchers have started to focus on the role of SV40 in regulating cell cycle control mechanisms. Lukas et al. (1994) showed that the interaction of SV40 large T antigen with Rb protein results in functional inactivation of Rb and a concomitant increase of cellular regulatory proteins like cyclin D1. Also, SV40 large T antigen might cause increased cell growth by repressing the expression of some cell cycle dependent growth inhibitory proteins like p65, which is a growth arrest specific gene product (Rose et al., 1992). In summary, these and other such findings support the hypothesis that interaction between SV40 large T antigen and cell cycle regulatory proteins play an important role in SV40 mediated cellular transformation (Evans

and Mueller, 1990).

4.0. ADENOVIRUS AND CANCER

The adenoviruses were first isolated from the cultures of adenoid tissues, hence the name. The human adenovirus genome consists of approximately 35 kilo base pairs of double stranded DNA which encodes for approximately 35 proteins. In human adenoviruses the entire viral genome is expressed in a regular and orderly fashion. The early viral genes and the corresponding proteins are the ones which play the most important role in the adenoviral mediated cellular transformation (Doyle and Crawford, 1994). There are six primary early transcripts which are converted to more than 16 early mRNAs which function analogously to the early gene products of SV40 and polyoma virus. In the adenoviral genome, the transforming ability resides in an early viral region called E1(Boyd et al., 1993; Wang et al., 1993). It is further divided into E1a and E1b. Jelinek et al. (1994) recently demonstrated that the tumor formation of adenovirus type 12 transformed rodent cells is controlled by region between CR2 and CR3 of Ad12 E1a. This segment between CR2 and CR3 has homology with a similar region in another highly

oncogenic adenovirus AD7 (Telling and Williams, 1993). The importance of this segment is further substantiated by the fact that it is entirely missing from the E1A gene of adenovirus type 5 virus which is non-oncogenic (Telling & Williams, 1993). Thus it is postulated that the oncogenic potential of Ad virus resides mainly in the region between CR1 and CR2 (Leclere et al., 1993; Mymeryk and Bayley, 1994). In addition to E1a and E1b oncoproteins, recently it has been shown that some other viral oncoproteins might also play some role in cellular transformation, either independently or in concert with some other viral proteins. For example, Javier et al. (1994) showed that the human adenovirus E4 open reading frame encodes a transforming protein which is required for the induction of mammary tumors in mice. The recent development of transgenic mice with adenovirus type 12 E1a/E1b genes might also help us to understand more clearly the mechanisms and role of adenoviral oncoproteins in the malignant cellular transformation (Belingieri et al., 1993; Ullrich et al., 1994).

4.1. Molecular Mechanisms By Which Adenovirus Plays a Role in Cellular Transformation

Although E1a can play a role in immortalization of

rodent and human cells, it needs other adenovirus E1b protein and/or other oncoproteins, like ras, for neoplastic transformation (Douglas et al., 1994). Possible mechanisms by which adenoviral proteins play a role in immortalization, cellular transformation and malignant change are discussed below.

4.1.1. Adenovirus and tumor suppressor protein

Adenovirus early proteins can cause functional inactivation of tumor suppressor proteins resulting in aberrant cellular growth (Shepherd et al., 1991; Teodore et al., 1994). E1a forms complexes with Rb protein (Guilhort et al., 1993) and E1b forms complexes with p53 protein (Grand et al., 1993; Chang et al., 1993). When E1b binds to p53 protein, it results in the abrogation of the p53 transactivation function (Va dereb et al., 1993). In addition, this interaction can also result in the release of transcription factors from their complexes with these tumor suppressor proteins (Fattaey et al., 1994). For example, E2F release of from Rb or p53 results in transactivation of several other factors which may play an important role in controlling the growth in the cells. E1a protein can also influence transcription by changing the phosphorylation

patterns of some of the cell cycle regulatory proteins (Dumont et al., 1993; Mymryk and Bayle, 1994).

Another possible way by which these adenoviral oncoprotein-p53 interactions can cause malignant transformation of cells is by modulating the apoptosis (programmed cell death) (Lowe and Ruley., 1993; Lowe et al., 1994). E1a protein may play an important causal role in immortalization of rodent cells but fails to neoplastically transform the cells possibly due to the induction of p53 mediated apoptosis (Subramanian et al., 1993; Sabbatani et al., 1995). Recently Chiou et al.,(1994) showed that BCL2 expression and possibly E1b expression by passes the induction of apoptosis by p53 and this might contribute to the oncogenic activity of the E1b protein. More studies are required to clearly understand the role of adenoviral mediated apoptosis in cellular transformation (White, 1993).

4.1.2. Adenoviral interactions with some other cellular proteins.

E1a protein can lead to increased expression of DNA polymerase alpha and can also interact with AP-1, TRIIIF, Heat Shock protein (HSP), P300 and p60 (cyclins) (Rosahl & Doerfler, 1992). The deregulation of cell cycle control due

to E1a mediated changes in cyclins might be an important mechanism of cellular transformation. In addition E1a protein can also increase the expression of fos, jun, myc as well as viral proteins like E1b, and E4 (Martin & Haung et al., 1994). Since E1a protein does not bind to DNA, it is probable that its effects on such a wide array of proteins are mediated indirectly, e.g., by alteration in the phosphorylation pattern of these proteins and/or by sequestering regulatory proteins (Peeper and Zantema, 1993).

In spite of current advances in understanding the molecular mechanisms of adenovirus induced cellular transformation, there are still many areas which need more research, e.g., the role of E1a protein in cell cycle control (Hinds et al., 1994), interaction of adenoviral proteins with the host immune system (Katz et al., 1994) and its precise role in the multi-step malignant transformation needs to be clarified (Boyd and Barrett, 1990)

5.0. HERPES VIRUS AND CANCER

The herpes viruses are a group of DNA viruses, some of which are strongly implicated in the causation of some human cancers. The herpes viral family is composed of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella

zoster virus, cytomegalovirus, Epstein-barr virus(EBV) and recently discovered human herpes simplex virus type 6 (HSV-6) and type 7 (HSV-7). Since EBV is most clearly implicated in human tumorigenesis, it will be dealt with in some detail.

5.1. EPSTEIN-BARR VIRUS AND HUMAN CANCER

EBV was the first virus to be recognized as playing a causal role in human tumor formation. Though the presence of viruses in pediatric lymphomas was described by Burkitt in 1958, it was Epstein & Barr who isolated viral particles from lymhoblastoid cell lines derived from patients with Burkitt's lymphoma. EBV is associated with infectious mononucleoses, Sjogren's Syndrome (Miyaska et al., 1994), lymphoproliferative disorders in immunosuppressed patients (Shibata et al., 1993), several human malignancies such as nasopharyngeal carcinoma, as well as B cell(Khan et al., 1993b; Lieboeitz, 1994; Katz and Witz, 1993) and T cell lymphoma (Albeck et al ., 1993; Miller et al., 1987; Shibata et al., 1994). A possible role for EBV in human tumors is demonstrated by the ability of the virus to immortalize B lymphocytes and epithelial cells in culture (Allday et al.,1993; Sinclair et al., 1994). Cheng et al. (1993) showed EBV viral gene expression during early

stages of B cell transformation. By the use of molecular and biochemical techniques, the EBV encoded genes and the gene products have been identified (Wang et al., 1987).

The genome of EBV is a double stranded DNA molecule of approximately 170,000 base pairs organized as regions of repeated DNA sequences. There are 6-12 tandem copies of a 500 bp units located at the end of linear genome (Robertson and Kieff, 1995).

Out of the several EBV proteins two EBV nuclear proteins (EBNA) namely Epstein-Barr Nuclear Antigen-1(EBNA-1) and Epstein-Barr Nuclear Antigen-2 (EBNA-2) (Grasseret al., 1993) and one membrane associated protein called Latent Membrane Protein (LMP) are strongly implicated in the etiology of human cancer (Sample et al., 1986).

5.1.2. EPSTEIN-BARR VIRUS NUCLEAR ANTIGENS AND CELLULAR TRANSFORMATION

EBNA-1 is viral nuclear protein that is expressed in virtually all EBV immortalized lymphocytes. Transfection studies using EBNA-1 expression vectors showed that only some of the cells expressing EBNA-1 were immortalized (Wang et al., 1987), suggesting that additional genetic events are needed (Gross et al.,1989). Recent studies indicate that EBNA-1 does

play a causal role in cellular immortalization in some type of cells. Using EBNA antisense oligonucleotide, Roth et al. (1994) demonstrated that proliferation of EBV immortalized cells can be inhibited by EBNA antisense oligonucleotides. These researchers showed that the exposure of EBNA-1 antisense oligonucleotide (codon 6-10 of EBNA-1) in EBV immortalized cells resulted in decreased expression of EBNA-1 protein and greatly decreased proliferation of these cells.

EBNA-2 is an acidic protein that can act as transcriptional activator of both viral and cellular genes. Cellular proteins that can be transcriptionally activated by EBNA-2 include the B cell activation antigens, CD21, CD23 (Wang et al., 1987) and the proto-oncogene c-fgr (Contreas-Bordin et al., 1991; Knutson, 1990). EBNA-2 can also immortalize rodent cells (Wang et al., 1987; Tong et al., 1994). EBNA-2 expression in Rat-1 cells confers the ability to grow in a medium with a low serum concentration. Tsui & Scubasch (1994) showed that EBNA-2 proteins form oligomer in vitro through a region encompassing amino acid 122-344. They further showed that this is the region which is required for B cell transformation.

Other EBNA proteins like EBNA-3 (Contreas- Bordin et

al., 1991), EBNA-4 (Silinis & Sculley, 1994), EBNA-5 and EBNA-6 (Allday et al., 1994) have also been shown to play a role in cell transformation, but the precise mechanism of their role is yet to be elucidated. Though EBNA proteins seem to be playing a causal role in cellular transformation of certain cell types, it is clear that further changes are required for malignant transformation (Chang et al., 1993; Evans et al., 1993).

In summary, all the EBV nuclear proteins appear to play a role in cellular transformation. There seems to be a need for additional genetic alterations for EBV mediated malignant transformation. The expression of EBNA proteins might interact with other regulatory proteins including tumor suppressor proteins, possibly in a cell specific manner. This might also involve components of signal transduction. Such interactions with the growth controlling processes can lead to EBNA mediated cellular transformation which in appropriate cells and conditions might progress to a malignant state.

5.2. LATENT MEMBRANE PROTEIN (LMP) AND CELLULAR TRANSFORMATION

Latent membrane protein (LMP) is the only viral membrane protein known to have the property of transforming human cells (Hu et al., 1994) and rodent cell lines like Rat-1

(Moorthy et al., 1993), and Balb/3T3 cell line (Wang et al., 1985). LMP is the most frequently detected EBV membrane protein in EBV related human cancers (Longnecker et al., 1992). Doyle et al. (1994) showed that in the absence of LMP, EBV was unable to transform cells suggesting that LMP is essential for EBV mediated cellular transformation. Some researchers have shown that EBNA-2 acting as transcriptional factor can transactivate LMP. Johansen et al. (1995) showed that EBNA-2 transactivated the LMP promoter and this transactivation is mediated by recently characterized cellular proteins Jk and Pu.1 (Robertson et al., 1995; Johannsen et al., 1995; Gross man et al., 1994). Besides EBNA-2, other EBNA proteins like EBNA-6 have been shown to induce LMP independently or in concert with EBNA-2 (Allday et al., 1993). Minarovitis et al. (1994) showed that methylation inhibits the activity of LMP. Recently, Crawford et al. (1995) demonstrated induction of LMP expression by interlukin 4 in EBV infected leukemic B Lymphocytes.

In-vitro cellular transformation studies have shown that LMP can transform rodent cells resulting in growth factor independent and anchorage independent phenotypes (Wang et al., 1987). Some of the anchorage independent clones when injected

into nude mice resulted in tumors (Baichwal & Sugden, 1988). Another way in which it is postulated that LMP can cause cellular transformation is by activation of the BCL2 gene resulting in the inhibition of programmed cell death (Henderson et al., 1991). Furthermore, due to its membrane association and phosphorylation of serine 313 and tyrosine 324 residues, it is hypothesized that it might also be interacting with some of the membrane associated receptor tyrosine kinase (Moorthy et al., 1993). LMP's expression in the cell can also increase the levels of interlukin 10 (IL-10) which is a strong B cell activating factor (Finks et al., 1994; Nakagomi et al., 1994). Wendel-Hansen et al., (1994) showed that certain cytokines can cause increased expression of LMP which in turn results in increased levels of IL-10. This cascade of cytokine-LMP-IL-10 activation might play an important role in the cellular transformation mediated by EBV (Miyazaki et al., 1993). Recently, Miller et al. (1995) showed that LMP induces expression of epidermal growth factor in some cells.

In summary, EBV infection is closely related to malignant transformation of human cells, both in vivo and vitro. EBNA proteins play an important role in immortalization of some types of human (e.g., B cells)and

rodent cells in culture. LMP, on the other hand, plays a role in cellular transformation to anchorage independence and growth factor independence. However, it is clear that additional genetic changes are required for the malignant transformation of EBV-transformed cells.

6.0. RETROVIRUSES AND CANCER

Retroviruses play an important role in certain cancers and studies with retroviruses gave important insights to the oncogenes (Vecchio, 1993). Retroviruses contain two large identical single stranded RNA molecules together with the enzyme reverse transcriptase, a RNA-directed DNA polymerase. After the virus is in the host cell, reverse transcriptase catalyzes the formation of a double stranded DNA from the RNA of the virus (Temin & Baltimore,1972). The enzyme possesses three enzymatic activities: 1)it copies RNA to give DNA-RNA hybrid; 2)it degrades the RNA in DNA-RNA hybrids; and 3)it copies the single stranded DNA to form a double stranded DNA. The resultant double stranded DNA is not an exact copy of the parental viral DNA. The sequences from the 5' and 3' end of each RNA molecule are combined and duplicated to form a long terminal repeat (LTR) at both ends of the double stranded DNA. This double stranded DNA then gets inserted into the host

genome to form a DNA provirus. In the growth cycle of the retrovirus, integration of the viral DNA in the host chromosome is obligatory in contrast to that of DNA viruses. Retroviruses are now generally classified as acute transforming viruses and non-acute transforming viruses, based on their potential to cause neoplastic transformation (Bouton & Parson, 1993).

6.1. NON-ACUTE TRANSFORMING RETROVIRUSES

Non-acute retroviruses cause animal tumors with long latency (Teich et al., 1984; Athas et al., 1994). Non-acute transforming viruses implicated in animal cancers include Murine Mammary Tumor Virus (MMTV) (Matsuzawa et al., 1995; van -Leeuwen and Nusse, 1995), Murine Leukemia Virus (MuLV)(Kozak et al., 1990), Friends Leukemia Virus (Tambourin et al., 1969), Maloney Leukemia Virus (Ihle et al., 1989), Avian Leukosis Virus (ALV)(Kung et al., 1981), and Feline Leukemia Virus (FeLV) (Besmer et al., 1983; Bergold et al., 1987)

6.2. MOLECULAR MECHANISM(S) INVOLVED IN NON-ACUTE RETROVIRAL CARCINOGENESIS

Non-acute transforming viruses are replication competent RNA viruses i.e., they carry sufficient information to generate all the protein required for virus production. They

can play a role in cellular transformation by a mechanism called Insertional Mutagenesis (Kung et al., 1991). The virus inserts into the host genome and this insertion is potentially mutagenic. This insertion can result in two potential changes: it can either damage some cellular gene(s) directly or it can influence the expression of cellular gene(s) by bringing them under the control of the regulatory elements in the viral genome (Vijaya et al.,1987; Kung et al., 1991). Mechanistically, the viral insertional mutagenesis can be of two types: promoter insertional activation or proviral enhancer activation (Trembley et al., 1992).

6.2.1. Promoter Insertional Activation

This process was first demonstrated by Hayward and colleagues in 1981. These researchers showed that in the B-cell lymphomas induced by Avian Leukemia Virus (AVL), the provirus was inserted at a specific location, i.e., in the vicinity of the c-myc proto-oncogene (Groudine and Weintraub, 1980; Hayward et al., 1981). Further investigation by other researchers showed that the proviral insertion led to the transcriptional activation of c-myc proto-oncogene expression by read through from the cryptic viral promoter in the ALV downstream LTR. Promoter insertional activation is also seen

in ALV induced erythroblastosis in chicken (Fung et al., 1983). Promoter insertional activation is also seen in the Muloney Leukemia Virus (MuLV) activation of c-myb proto-oncogene (Shen-Ong et al., 1987; Kanter et al., 1988).

In summary, as a consequence of the proviral insertion in the vicinity of a cellular proto-oncogene, high efficiency transcription from the viral LTRs leads to the over expression of the cellular proto-oncogene product resulting in unregulated growth (Groudine and Weintraub,1980). This unregulated growth in concert with other genetic changes can finally lead to malignant transformation of cells.

6.2.2. Proviral Enhancer Activation

In some virus-induced tumors, the model of proviral promoter insertion described above was not seen. In tumors like AVL-induced B-cell lymphomas (Payne et al., 1982) and MMTV-induced mammary tumors (Nusse and Varmus, 1982) the provirus was inserted next to proto-oncogenes in the opposite configuration. Instead of the classical promoter insertion upstream of the cellular proto-oncogene, the provirus was inserted either in the opposite transcriptional orientation or downstream of the cellular proto-oncogenes (Payne et al., 1982; Nusse and Varmus, 1982; Cuypers et al., 1984). Even in

such orientations there was increased expression of the proto-oncogenes (Groudine and Weintraub,1980). The explanation offered is that the proviral enhancer elements or sequences in the retroviral LTR were responsible for activating the transcription from the proto-oncogene's own promoter. Enhancers are cis acting DNA elements which can activate the adjacent promoters in orientation, distance and position independent fashion. Recently, Morrison (1995) showed that misincorporation and/or recombination can alter the enhancer structure resulting in a generation of more pathogenic variants.

The study of non-acute transforming retroviruses has helped to identify several proto-oncogenes that were never transduced or captured by the acute transforming viruses e.g., pim-1 in MuLV induced lymphomas (Cuypres et al., 1984), int-1, int-2 and int-3 in MMTV induced mammary tumors (Nusse et al., 1984; Roelinink et al., 1992). Proviral LTR activation of the proto-oncogenes also explains in part the long latency of non-acute transforming virus induced tumors. The retrovirus DNA integrates at multiple sites in the host DNA. The very likelihood that the proviral promoter insertion will be in the proximity of cellular proto-oncogenes is low.

Hence, multiple rounds of the viral infection which requires a long time interval are required for the chance insertion of a provirus next to an appropriate cellular proto-oncogenes.

7.0. ACUTE TRANSFORMING RETROVIRUSES

Acute transforming viruses are non-replication competent retroviruses which are derived from the non-acute retroviruses. They carry oncogenes which endow them with the potential to rapidly induce tumors in animals (Vogt, 1987). Some of the important acute transforming viruses are Rous Sarcoma Virus, Feline Sarcoma Virus and Simian Sarcoma Virus. Despite the fact that retroviruses induce cancers in a wide range of animals and birds, no retrovirally-borne gene or gene product has yet been demonstrated to be causative of human cancer.

7.1. MOLECULAR MECHANISMS OF ACUTE TRANSFORMING RETROVIRAL CARCINOGENESIS

When a non-acute retrovirus infects a cell and in the process captures a cellular proto-oncogene, it becomes an acute transforming virus (Stehelin et al., 1976). A recombination event between the cellular and retroviral genome results in the insertion of the cellular gene into the viral genome and a loss of an equivalent length of the viral genome.

The transduced gene is then under the influence of the viral promoter which can result into aberrant expression. Since the cellular gene is now being regulated by a viral promoter, its expression is independent of the site of the proviral insertion within the chromosomal DNA. Additionally, during the process of transduction, the cellular proto-oncogene might undergo structural alterations like point mutations, gene rearrangements, deletions and fusions. These genetic alterations alone might convert cellular proto-oncogenes into oncogenes or they may enhance their virulence (Bishop and Varmus, 1984). In the case of the cellular src genes (c-src) a stretch of a few nucleotides was lost during the process of transduction resulting in many fold higher expression of src in virally infected cells. Specific point mutations might also result in activation of certain cellular proto-oncogenes as seen in the case of ras and neu (Barbacid 1987). In each case, the acutely transforming virus plays a causal role in the carcinogenesis by introducing an activated protooncogene into a cell resulting in some type of aberrant cellular growth.

D. IONIZING RADIATION AND CANCER

Ionizing radiation (x-rays and gamma rays) and

particulate radiation(alpha particles, beta particles, protons and neutrons) are carcinogenic in humans, and some of them can cause neoplastic transformation of some cells in culture (Rhim , 1993; Reinhold et al., 1996). Epidemiological and experimental evidence strongly supports the view that accidental and experimental exposure to ionizing radiations may play a causal role in some types of human cancers (Armitage and Doll, 1985). The first neoplasm attributed to radiation was an epidermoid carcinoma on the hand of a radiologist (Frieban et al., 1902). Within the next few years, 94 cases of skin cancer had been reported to be attributed to exposure to radiation among physicians, radium handlers and x-ray technicians in America, England and Germany (Hesse et al., 1910). These were predominantly squamous and basal cell carcinomas but a few fibrosarcomas were also seen. The first association of hematopoietic cancers like leukemia and exposure to ionizing radiation was reported by von Jaggie et al., 1911. Since then, several hundreds of radiation-induced leukemia have been reported in literature (Cronkite et al., 1960). Epidemiological studies also confirmed the association of leukemia with ionizing radiation exposure (Armitage & Doll, 1985; Fraumeni et al., 1990). The high

incidence of cancer in radium dial painters was first suspected by Martland in 1901 (Fry, 1985). The dial painters used to put their brushes between their lips to shape them when applying the luminous paint to the watch and clock dials. In doing so they ingested radium. In 1929, Martland first reported the high incidence of bone tumors, osteosarcomas, in these dial painters (Martland and Humphries, 1929; Wagener et al., 1960). In addition to the osteosarcomas commonly seen in these patients, an increased incidence of other tumors like carcinoma of paranasal sinuses, mastoid sinuses and fibrosarcoma was also reported. A high incidence of bronchogenic carcinoma in radioactive mine workers in Central Europe and the Rocky Mountain region of United States of America has been attributed to inhaling radon and its radioactive disintegration products.

Experimental studies also provide evidence of association of exposure to ionizing radiation and cancer in humans. Soon after the detection of the first radiation-induced human cancer, experiments performed on animals showed that neoplasms of virtually any kind could be induced by the exposure to ionizing radiation. Furthermore, ataxia telangiectasia, an autosomal recessive syndrome, is characterized by increase

sensitivity to ionizing radiation and an increased predisposition to cancer (Morgan et al., 1968). In-vitro cellular transformation studies using rodent and human cells (Namba et al.,1981, Namba et al., 1993; McCormick et al., 1995; Little and Novak, 1990) showed that cells exposed to ionizing radiations could be transformed. Some of these transformed cells formed foci and/or anchorage independent colonies. On occasion, cells from these assays formed tumors in athymic mice (Rhim et al., 1985; Reinhold et al, 1996)

1.0. MOLECULAR MECHANISMS INVOLVED IN RADIATION-INDUCED CARCINOGENESIS

Ionizing radiation causes a variety of molecular lesions in essentially every cellular organelle and subcompartment (Weichselbaum et al., 1991; Fornace, 1992). The lesions most detrimental and important in carcinogenesis are those in the DNA (Ward, 1995). Several biochemical and molecular studies have shown that radiation-induced lesions are produced either by a direct deposition of the energy in the DNA or indirectly by the interaction of DNA with the radiation-induced free radicals like H_2O_2 (Ward, 1995). Both direct and indirect effects of ionizing radiation produce a variety of DNA lesions like sugar and base damage (Teoule, 1987; Osman et al., 1991;

Cadet, 1994), single strand breaks (Ward, 1995) and the double strand breaks (Nelson and Kastan, 1994; Cole et al., 1980; Paquette and Little, 1992; Ward, 1990). As a result of ionizing radiation-induced DNA damage, cells may survive or they die due to necrosis or apoptosis. Surviving cells may have received no damage or received damage which was completely repaired, incompletely repaired, not repaired or misrepaired (Elkind, 1985; Teoule, 1987). The complex repair mechanisms involved in the repair of radiation-induced DNA lesions involves checkpoints located in G-1 and G-2 and many DNA repair proteins (Lehman and Norris, 1989). In the last few years several x-ray cross complimenting (XRCC) genes have been identified and localized to several different human chromosomes (Lehman, 1995). Defective cell cycle checkpoints, the failure of a cells to undergo complete DNA repair as well as misrepair of the DNA lesions can result in mutations (Kuerbitz et al., 1992). A detailed understanding of the molecular and biochemical mechanisms involved in ionizing radiation injury, DNA repair, cell death and cell survival will help us to clearly delineate the how ionizing radiation induces human cancer (Lee et al., 1994; Ward, 1995).

E. ULTRAVIOLET RADIATION AND CANCER

Ultraviolet radiation from the sun has been implicated in the etiology of human Squamous Cell Carcinoma (SCC) and Basal Cell Carcinoma (BCC) (Urbach et al., 1978; Scotto & Fears, 1978; Brash et al., 1991; Yuspa, 1994). Ultraviolet radiation is classified as UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). Visible light is in the region between 400 nm and 780 nm. Since atmospheric ozone absorbs essentially all of the UVC and most of the UVB, the spectrum of UV that reaches the earth primarily consists of UVA (90-99%) and UVB (1 to 10%). Depletion of stratospheric ozone in the last few years is postulated to result in a higher incidence of SCC and BCC (Kerr and McElroy,1993; Fears and Scotto, 1983; Lloyd, .1993).

The wavelength of UV radiation also determines the depth of the skin which can be penetrated by the photon. For example, one-third of UVA (315-400 nm) penetrates the skin to a depth of 0.1 mm. However 99% of UVB is absorbed by the upper 0.03 mm of the epidermis. Most evidence indicates that the UVB accounts for most of the biologic damage caused by solar UV radiation (Urbach et al., 1978; Epstein, 1978), although a few studies suggest a possible role of UVA in

cellular transformation (Forbes, 1982)

1.0. Biological Effects of Ultraviolet Radiation

Ultraviolet radiation exerts its biological effects mainly by its interaction with cellular DNA (Jones et al., 1987; Zolzer and Kiefer, 1989). There are two pathways: photons with energy in the UVB spectrum are absorbed directly by DNA resulting in the formation of photo products (Moan & Peak, 1989; Peak & Peak, 1989) and the photons with energy in UVA spectrum causes reactive oxygen species resulting in single strand breaks and DNA protein cross links (Tyrell & Kyse, 1990). Although UV radiation-induced immune suppression may play some role in human carcinogenesis (Kripke, 1994; Kripke et al., 1996), most investigators consider this to be a minor role at most and so it will not be discussed.

2.0. Role of Ultraviolet Radiation

Experimental and epidemiological studies strongly implicate UV radiation as a cause of skin cancer (Scotto and Fears, 1978; Scotto et al., 1981; Slaga, 1982)). Findlay in 1928 was the first to demonstrate that the chronic exposure to UV radiation resulted in cancer in albino mice (Findlay, 1928). Using the initiaton-promotion model of cellular transformation , several workers have shown that ultraviolet

radiation acts as a "complete carcinogen". In this two step model ultraviolet radiation was shown to be acting as an "initiator" in causing DNA damage and as a "promoter" causing the clonal expansion of the initiated cell (Forbes,1981; Urbach et al., 1978).

Animal research focused on two main aspects of UV-induced carcinogenesis: to develop an action spectra for carcinogenesis and secondly to determine the relative contribution of the different UV wavelengths in the causation of tumors(Suzuki et al., 1981). Studies in 1970's and 1980's proved that UVB is most important in UV-induced carcinogenesis. Davies and Forbes(1986) showed that in mice UV-induced squamous cell carcinoma and the increased incidence of mortality in these mice was due to UVB. Urbach (1989) was the first to suggest that UVA can augment the carcinogenic effects of UVB.

The role of UV radiation in skin carcinogenesis is clearly substantiated by the higher incidence of skin cancers in individuals with the rare genetically-transmitted disease, xeroderma pigmentosum (XP) (Robbins et al., 1974; Patton et al., 1984; Cleaver 1990; Jones et al., 1992a). This disease is characterized by clinical hypersensitivity to ultraviolet

radiation and by a defective DNA repair mechanism (Bohr and Wasserman, 1988; Hanawalt & Melon, 1993; Evans., 1993; Kraemer et al.,1989; Setlow et al., 1969; McCormick & Maher, 1981; MacGregor et al., 1992). The initial description of XP was made by Moriz Kaposi in Germany in 1870. The first American case of XP was described by Taylor in 1878 (Taylor, 1878). So far more than 1000 cases have been reported in the literature. The recent generation of mice lacking the murine homologue of the genes defective in XP patients shows that such mice show a high susceptibility to ultraviolet radiation induced carcinogenesis (Sands et al., 1995; de Vries et al., 1995). In short, experimental studies on the cells of XP patients as well as from mice with similar defects provides valuable insight into the role of UV and DNA repair in the etiology and pathogenesis of UV-induced skin carcinogenesis (Maher et al., 1976; Maher et al., 1982; Evans et al., 1990; Lambert & Lambert, 1995).

3.0. Molecular Mechanism of Ultraviolet Radiation Carcinogenesis: UV-Induced Photo Lesions

Two main types of UV photo products arise due to direct absorption of UVB radiation by DNA: cyclobutane pyrimidine-pyrimidine dimers and (6-4) pyrimidine-pyrimidones (Taylor,

1987) The most frequent photo product is the cyclobutane dimers. With UVB, there are about three times more cyclobutane dimers produced than 6-4 products (Mitchell, 1989; Mitchell et al.,1991). The frequency of UVB-induced cyclobutane dimers in DNA follows the pattern TT>TC>CT>CC (Gordon and Haseltine, 1982; Bouree et al., 1985), with TT lesions occurring 2-3 times more frequently than the CC lesions. Besides these two major classes of photo products, there are several other photo lesions like thymine glycols (Hariharan & Cerutti, 1977), cytosine photohydrates (Weiss & Duke et al., 1987), purine photo products (Gallagher et al., 1989), DNA strand breaks (Miguel & Tyrell, 1983; Tyrell et al., 1974; Cadet, 1994), DNA protein cross links (Kolomiitseva et al., 1994) which occur at a low frequency. These minor types of UV-induced photo lesions are not thought to be important in the process of carcinogenesis.

4.0. GENETIC CONSEQUENCES OF PHOTO PRODUCTS: UV-INDUCED DNA MUTATIONS

DNA photoproducts caused by UV radiation are removed and the DNA resynthesized by repair mechanisms (Lambert & Lambert, 1995). If the lesions are not removed before DNA replication takes place, mutations can be generated by a failure to insert

the correct nucleotide across the lesion (Keohavong et al., 1991; Maher & McCormick, 1989; McGregor et al., 1991; Tung et al., 1996).

In 1991, McGregor et al. found that the unique DNA lesions induced by UV radiation produced a distinctive mutation pattern e.g., a " UV-signature ". He found that in human cells, C-T transitions and CC-TT double base substitutions are the typical mutations associated with UV exposure. These characteristic mutations were hypothesized to arise due to misincorporation of A residues at the non-instructional lesion sites. According to the so called "A" rule, when the DNA polymerase comes across lesions on DNA that it cannot interpret, it inserts A residues. Hence T--T dimers which are the most frequent photo product are non-mutagenic. However C-C dimers give rise to C-T mutations. It should be noted that that some workers lately are questioning the applicability of the "A" rule in UV-induced mutations (Sage, 1993). Using C-T transitions and CC--TT double base substitutions as a signature for UV-induced mutations, several workers have examined skin tumors to determine whether such mutations are present in genes thought to be responsible for cancerous growth (Brash et al., 1991; Dumaz et

al., 1991). These results show that presence of UV signatures in proto-oncogenes and tumor suppressor genes in skin tumors suggesting that UV plays a causal role in their etiology (Nartaraj et al., 1995; Ananthaswamy and Kanjilal,1996).

5.0. Role of Oncogenes in Ultraviolet Radiation-Induced Cellular Transformation

Cellular oncogenes are reported to be activated in a variety of human and rodent tumors (Bishop et al., 1987; Balmain & Brown, 1988; Weinberg, 1985). The normal cellular counterpart of oncogenes, proto-oncogenes, typically play a role in regulation of normal cellular growth. Exposure of the cells to carcinogens can convert the proto-oncogenes to oncogenes. Typically this results in either a structurally abnormal gene product with increased activity or a marked increased expression of the normal gene product. There is in-vivo and in-vitro evidence available that both these phenomena occur in cells (Barbacid, 1987 ; McCormick et al., 1991; Pierceall et al., 1992).

As an example, the role of ras oncogene in this cellular transformation will be discussed. In-vivo and in-vitro studies indicate that ras is one of the most common oncogene mutated in skin cancers (Quantanilla et al., 1986; Elder,

1990). Evidence is available implicating ras oncogene both in the earlier and later steps of malignant transformation (Balmain et al., 1989). Ananthswammy et al. (1990) showed that genomic DNA isolated from non-melanoma skin cancers from sunlight exposed parts of the body contained an activated c-H ras oncogene. When these activated *ras* genes were transfected into NIH3T3 cells, malignant transformation resulted. Analysis of human skin cancer (in melanoma and non-melanoma) have revealed point mutations in one or the other of the three members of the *ras* gene family (K-ras, N-ras and H-ras) (Pierceall et al., 1991; Van'T Veer et al., 1989). In most skin tumors ras mutations were found at codon 12 or 61, opposite the dipyrimidines in the other strand. This suggests that in skin cancer UV-induced photo products (dimers or 6-4's) are actively involved in the induction of mutation in the ras proto-oncogene oncogenes (Ananthaswamy & Pierceall, 1990). Ras gene amplification has been reported in human squamous cell carcinoma (Pierceall et al., 1991), melanomas (Funato et al., 1989) and in squamous cell carcinoma of skin in XP patients (Suarez, 1989; Suarez et al., 1989; Steingrimsdottir et al., 1995). In most of cases ras gene amplification was accompanied by point mutations, but in some

cases gene amplification was present in unmutated sequences (Boss, 1989; Pierceall et al., 1991).

Ras mutations in melanoma cell lines from XP patients with cutaneous melanomas have been detected (van'T Veer et al., 1989). Most of these mutations were at codon 12, 13 or 61. These results suggest that an activated *ras* gene plays a role in the transformation of melanocytes to melanoma (Pierceall et al., 1991).

In-vitro experiments confirmed the observations of UV-induced DNA damage in animals or human skin cancers. Using NIH 3T3 cells, Bezlepkin et al. (1991) showed a dose-dependent increase in UV-induced fibroblast transformation. Most of these UV-induced transformants contained mutations at codon 12 or 61 in a ras gene opposite a run of pyrimidines indicating the causal role of UV in this cellular transformation. Cells cultured from mouse skin tumors induced by UV showed similar results (Kanjilal et al., 1993).

UV-induced skin tumors in mice have also been found to have *ras* mutations (Strickland et al., 1985). Sutter et al. (1993) showed ras gene activation in UVB-induced epidermal neoplasia in mouse skin. Husain et al. (1990) found that UVB-induced mouse papillomas and carcinomas expressed H-ras

protein at 3-5 fold higher levels than the controls. They showed that when DNA from the UV-induced mouse carcinomas was transfected to NIH 3T3 cells, transformed foci resulted. This result was not seen when DNA from UV-induced papilloma was transfected. These and other experiments done by Balmain and co-workers indicate that additional genetic changes (oncogene activation and/or tumor suppressor gene inactivation) are required for the UV-induced malignant transformation.

5.0. Role of Tumor Suppressor Genes in UV-Induced Malignant Transformation

Tumor suppressor genes play a causal role in cancer when they no longer function to restrain cellular growth (Deppert,1994). This requires the loss of function of both alleles (Vogelstein and Kinzler,1992b). Small or large deletions, chromosomal rearrangements, as well as point mutations all can lead to the loss of the ability to make functional protein (Fearon & Vogelstein, 1991).

The *p53* gene is the most frequently mutated gene in human cancers (Levine et al., 1991; Stampfer et al., 1993). It will be discussed in some detail as a prototype suppressor gene to illustrate the possible molecular and biochemical mechanisms by which such genes play a role in the regulation

of cellular growth and consequently in carcinogenesis.

5.1. P53 Tumor Suppressor Gene and Protein

The p53 tumor suppressor protein was first identified because it co-precipitated with SV40 large T antigen and was assumed to be a viral associated protein (Linzer & Levine, 1979; Lane & Crawford, 1979). Subsequently, transfection of the p53 gene into cells with an activated ras led to malignant transformation of the cells indicating p53 was an oncogene (Hinds et al., 1989). In 1983, Finlay and colleagues demonstrated that in fact the *p53* gene used in the cell transformation studies was a mutant version of normal p53. In 1991, it was found that the wild type p53 gene was in fact a tumor suppressor gene (Levine et al., 1991). When the wild type p53 gene is mutated, so that either no p53 protein is synthesized or that the protein sybthesized is non-functional, it causes increased cellular proliferation and aberrant cellular growth (Levine et al., 1991; Chang et al.,1994).

5.2. P53 tumor suppressor gene.

The *p53* gene is located on chromosome 17p13.1 in humans and chromosome 11 in mice (Benchimol et al., 1985). It consists of 11 exons which code for a protein of 393 amino acids in humans and 390 amino acids in mice (Lamb & Crawford,

1986). The first exon of the *p53* gene is non-coding. There are several highly conserved exons of which exons 4-7 are the most conserved ones. Two-thirds of the missense mutations are found at specific bases in these exons which are referred to as hot spots. The levels of *p53* mRNA are low in the normal cells but they are frquently elevated in transformed cells.

5.3. P53 TUMOR SUPPRESSOR PROTEIN

The *p53* gene encodes for a 53 kd nuclear phosphoprotein . The phosphorylation pattern of p53 regulates its function in a cell cycle dependent fashion. It is hypophosphorylated in early G1 and becomes phosphorylated by cdc2 kinases as the cell enters S phase(Bischoff et al.,1990). The N-terminus (residues 1-75 in humans) of the protein is very acidic and highly charged. The C-terminus (residue 319-393) is a proline rich hydrophobic region (Soussi et al., 1990; Donehower et al., 1993). The middle region (residue 75-110) is a basic DNA binding domain and contains serine residues phosphorylated by cdc2 kinase and casein kinases (Funk et al., 1992; El-Deiry et al., 1992).

5.4. Mechanism of Inactivation of p53

5.4.1. Gene mutations.

Missense mutations in *p53* gene can result in its

inactivation (Greenblatt et al., 1994). Missense mutations in one allele and subsequent loss of the remaining allele by genetic or epigenetic events can result in the loss of p53 suppressor functions. Other genetic alterations like gene rearrangements, chromosomal loss, deletions, etc. can also result in loss of one or both of the alleles of p53. Mutations can inactivate p53 in two ways. *p53* mutations can result in the loss of the p53 functions directly or the mutant p53 protein can oligomerize with the wild type p53 protein in transdominant fashion resulting in the loss of the wild type p53 activity (Lane, 1992).

5.4.2. p53 Protein and viral protein interaction.

SV40 large T antigen, adenoviral E1b and human papilloma virus E7 oncoproteins can interact with the p53 protein. These viral oncoproteins interact with specific domains of the p53 protein resulting in the loss of the p53 binding to DNA. This results in abrogation of transcriptional activity of the p53 protein. Alternatively,the interaction of p53 protein with the specific viral proteins noted above may lead to ubiquitin-mediated degradation. Both situations cause the loss 0f p53 suppressor functions (Band et al., 1995).

5.4.3. P53 and cellular protein interaction.

Some cellular proteins like the Minute-Double-Minute 2 (MDM2) protein can bind to p53 protein resulting in its inactivation. MDM2 gene amplification is seen in some sarcomas (Fornace, 1992) and some uroepithelial carcinomas (Habuchi et al., 1994). MDM2 is one of the cellular proteins which is transcriptionally activated by p53 protein. Elevated levels of MDM2 protein can interact in turn with p53 resulting in its inactivation (Cordon-cardon et al., 1994; Hind et al., 1994). Kastan and coullegues in 1994 demonstrated that MDM2 overexpression can inhibit p53 function in a known mammalian cell cycle checkpoint in G1 phase of cell cycle. They suggested that MDM2 might function in an autoregulatory feedback loop with p53 resulting in determining the length and severity of p53 mediated cell cycle arrest after radiaion damage (Chen et al., 1994).

In summary, there is an auto regulatory loop in which the wild type p53 protein transcriptionally transactivates the MDM2 gene.The MDM2 protein synthesized can bind to wild type p53 protein resulting in functional inactivation of p53 protein (Juven et al., 1993; Meltzer, 1994). Further study is required to fully understand the role of p53-MDM2 interaction in regulating the cellular growth in cells.

5.5. THE ROLE OF P53 TUMOR SUPPRESSOR PROTEIN IN CANCER

The p53 protein is one of the most extensively studied proteins in humans (Finlay, 1992). Diverse cellular functions have been attributed to the p53 protein (Unger et al., 1993). The role of p53 protein in cellular transformation correlates best with its ability to act as a transcriptional factor (Raycroft et al., 1990). The p53 protein is a DNA binding protein which binds to tandem copies of consensus sequence which is comprised of two copies of 5'-PuPuC (A/T) (T/A)GPyPy-3' separated by 0-13 bp (Kern et al.,1991; El-Deiry et al.,1992). p53 protein as such or in concert with other transcriptional factors binds to the promoters of the genes with this concensus binding sequence (Field & Jang, 1990; Raycroft et al., 1990), resulting in transcriptional activation of these genes. p53 protein can also bind to some promoters which do not have the p53 binding site resulting in transcriptional inactivation of such genes. WAF/CYP1/SIDII/P21 and GADD45 are two genes which are transcriptionally activated by the p53 protein (Kastan et al.,1992; Gujuluva et al.,1994; Michelli et al., 1994; Smith et al., 1994)

5.6. THE ROLE OF P53 IN ULTRAVIOLET RADIATION-INDUCED MALIGNANT CELL TRANSFORMATION

Several in-vivo and in-vitro studies suggest that p53 gene plays an important role in UV-induced carcinogenesis (Reiss et al., 1992). Mutational analysis of *p53* gene mutations in humans and animals have revealed the presence of hot spots in p53 gene for UV-induced mutations. Ten *p53* gene mutational hot spot areas have been identified in human skin cancers (151-152, 177, 196, 245, 248, 258, 278, 186, 194, 342). The distribution of the two major UV lesions in p53 gene indicates the highest frequency of mutations at codon 286 followed by codons 151 and 278. These three mutation hot spots are rarely involved in internal malignancies (Zeigler et al., 1993; Tornaletti et al., 1993). In human skin fibroblasts, Amstad et al. (1994) showed UV-induced mutagenesis hot spot in *p53* gene were at codons 248 and 249. Brash et al. (1991) detected p53 mutations in 58% of human squamous cell carcinomas. Recent studies have shown a much higher frequency (80%-100%) of p53 mutations in human skin cancers (Kanjilal et al., 1995). In non-melanoma skin cancers (basal cell carcinoma and squamous cell carcinoma) as well as in acanthosis keratosis, a UV induced skin lesion

thought to be precancerous, most of the *p53* mutations mainly were C-T transitions and/or CC--TT double base substitutions (Zeigler et al.,1993, 1995; Reed et al., 1993; Van Der Reit et al., 1994; Pierceall et al., 1991; Nelson et al., 1994; Nakazawa et al., 1994; Moles et al., 1993; Kress et al., 1992). These results reinforce the view that UV radiations play a direct role in skin carcinogenesis (Kanjilal et al., 1993a; Dumaz et al., 1994).

It not yet clear that *p53* mutations are early or late events in the process of UV-induced malignant transformations. Brash et al. (1994) indicated that UV-induced *p53* mutations are an early event. Campbell et al. (1993) suggested that p53 mutations precede tumor invasion in the squamous cell carcinoma of the skin. Ziegler et al. (1994) showed that *p53* gene inactivation abrogated the p53-dependent apoptosis of skin cells. These researchers proposed that *p53* besides acting as guardian of genome in cells, also acts as a guardian of the skin tissue in skin in response to DNA damage. Zeigler et al. (1994) proposed that UV can act both in the early steps in actinic keratosis (acting as initiator) and in the later steps (acting as promoter) in skin carcinogenesis these results in human are substantiated by studies on mouse skin

(Kanjilal et al., 1993b). Studies in the NMSC of XP patients also exhibit a UV signatures in the *p53* gene. In addition, p53 mutations were found at high frequency in the non-melanoma skin cancers(NMSC) of xeroderma pigmentosum patients. Sato et al. (1993) showed that five of eight NMSC studies in XP patients had p53 mutations mainly C-T transitions and CC-TT double base substitutions. Dumaz et al. (1994) showed that 40% of UV-induced skin cancers from XP patients had *p53* mutations. Dumaz et al., (1994) also demonstrated that 100% of the *P53* mutations were targeted at py-py sites and that 55% of these are tandem CC--TT transitions which are considered signature mutations of UV-induced lesions.

Dumaz et al., (1994) also demonstrated that nearly all (95 %) of the *p53* mutations in XP are located on the non-transcribed strand. Several other researchers have shown that UV-induced p53 mutations occur preferentially on the non-transcribed strand indicating a presence of preferential repair of the transcribed strand in XP patients (Evans et al., 1993; Ford et al., 1994). Besides, Tornalltti & Pfeifer (1994) found slow repair of pyrimidine dimers at p53 mutation hot spots in skin cancer suggesting that inefficient DNA repair might also contribute to the mutation spectra in UV-

induced skin cancer.

5.8.The role of P53 in Malignant Melanoma

The role of UV radiation induced *p53* mutations in the etiology and pathogenesis of malignant melanoma is not as clear (Brozena et al., 1993). Research has shown that the over expression of the p53 protein is a late event in melanoma skin cancers (Llassam et al.,1993). These researchers also showed that *p53* mutations occur in less than 10 % of melanoma patients (Llassam et al., 1993). In some cases of malignant melanoma,the MDM2 cellular protein was over expressed (Momand & Levine, 1991; Perry et al., 1993).

Recently another cell cycle regulated protein p16/MTS1 has been implicated as a causal factor in malignant melanomas (Kamb et al., 1994). Somatic mutations and homologous deletions were found in several tumor derived cell lines (Nobori et al., 1994). Liu et al. (1994) showed that in 14 out of 18 human nelanoma cell lines, the p16 mutations detected were C-T transitions at dipyrimidine sites. Two other melanoma cell lines carried CC--TT double base substitutions. These UV signature mutations imply that UV is involved in the pathogenesis of melanoma. However, the fact that melanoma occurs in the sunlight exposed and non-sunlight

exposed areas of the body indicates that other factors might be involved in the etiology of malignant melanoma (Kamb et al.,1994).

In summary, UV induces unique signature mutations (C--T and CC--TT) at dipyrimidine sites. The fact that these types of mutations are very rare in most cancers but are common in the oncogenes and/or the tumor suppressor genes of human and mouse skin cancers is evidence that the tumors arise as a result of DNA damage from UV radiation (Ananthaswamy & Pierceall, 1990; Brash et al., 1994; Vogelstein and Kinzler,1992b).

E. MULTI-STEP MALIGNANT TRANSFORMATION

Cancer is now considered to result from a multi-stepped process involving the activation of oncogenes and/or inactivation of tumor suppressor genes (Farber, 1984; Fearon & Vogelstein, 1991; Marshall, 1991). These genes can be altered in their structure and function by exposure to mutagenic carcinogens and/or by spontaneous mutations. It is proposed that activation of an oncogene or loss of expression of a tumor suppressor gene in a cell will give that particular cell some growth-adwantage over the other cells allowing clonal expansion of these mutant cells. The clonal expansion

of these cells increases the likelihood that a second mutation will occur in one of the progeny cells. This will bring the cell with mutations in two cancer related genes a step closer to being malignant. The clonal expansion of doubly-mutated cell increases the likelihood that the third mutation will occur in one of the progeny cells. This clonal expansion and sequential mutations will result finally in a cell which has acquired all the required number of changes needed for the malignant transformation. Clonal expansion of this cell results in an overt cancer (McCormick & Maher, 1994).

The number of genetic alterations needed for any human cell to become fully malignant is not known. The specific genes that must be mutated to generate a tumor cell are surely different in different cell types. It is also clear that childhood tumors like retinoblastoma require fewer changes as compared to adult tumors (Knudson et al., 1991; Vogelstein et al., 1991; McCormick & Maher, 1994). Various studies in animals and humans suggest that the number of changes required for complete malignant transformation of adult tissues range from six to nine (Vogelstein et al., 1991; McCormick et al., 1994).

One of the many problems in studying the molecular

mechanism of carcinogenesis is this that most of the studies to detect cancer related genetic alterations in humans are done on overt tumors. The cells in these human tumors (epithelial or mesenchymal) have already undergone multiple genetic changes in the process of transformation. The consistent detection of an activated oncogene or inactivation of a tumor suppressor gene in a specific tumors strongly suggests that it is playing a causal role in the tumorigenesis of the cells. However, the specific function of a gene can not be demonstrated from such findings. For these reasons, it is difficult to study the individual steps or the genetic chenges involved in the tumorigenesis process using human tumors. Most of the studies carried out so far to study the genes involved in malignant transformation of human cells involve placing cancer-related genes in murine cells like NIH 3T3 cells. This cell system provides us with valuable information about the actions of oncogenes. However, the changes already acquired by the NIH3T3 cells cannot be readily studied in these cells making it difficult to ascertain all the individual steps involved in carcinogenesis.

F. THE MSU-1.1 HUMAN FIBROBLAST TRANSFORMATION SYSTEM

Many workers have tried to establish in vitro cell

transformation systems using human cells, particularly using human fibroblasts to study the molecular changes involved in multistep carcinogenesis. In general, human fibroblasts are easy to be grown in culture and exhibit high cloning efficiencies (McCormick and Maher,unpublished observations). Though several workers have been successful in causing normal human fibroblast to acquire different transformed properties, these cells have not been succesfully transformed in culture to malignant cells as pointed out by McCormick and Maher (1988). One of the reasons they postulated for this difficulty is that normal human fibroblasts have a limited life span in culture and it is not enough to allow the cells to acquire the requisite number of changes for malignant transformation. Normal human cells in culture can only undergo two sequential clonal selections before entering into crisis and senescence (McCormick & Maher, 1988).

In view of such difficulties, McCormick and Maher and colleagues decided to work on an alterative method to study multi-step malignant transformation. They began their studies by establishing human fibroblast system in which genotypically and phenotypically normal cells are first immortalized and then carried step-wise forward. At each step, the cells were

selected for phenotypic changes like the ability to grow in agarose or in serum-free medium. The sequential clonal expansion of cells that had acquired the various properties finally yielded malignant cells.

Using the gene transfection approach of Weinberg and colleagues, McCormick, Maher and colleagues began by developing an immortal human fibroblast cell strain. The parental cell line was obtained from the foreskin of neonate and termed LG1. LGI cells are normal, diploid human fibroblasts with normal growth dynamics (Morgan et al., 1991). LG1 cells were transfected with a plasmid carrying v-myc oncogene and a selectable marker neo. Cells were selected for the drug resistant colonies. A clonal cell population that expressed v-myc protein was identified and propagated in culture for several months (McCormick et al., 1995). The cells entered crisis and finally senesced. A small group of cells, most probably a clone, was found replicating in the senescing population. This eventually gave rise to a MSU-1.0, a diploid, immortal human fibroblast cell strain with stable diploid karyotype. A spontaneous variant of MSU-1.0 cells with growth advantage arose and overgrew the MSU-1.0 cells in culture. These cells were designated MSU-1.1. MSU-1.1 cells

grow moderately well in serum free medium, form very small size colonies at low frequency in agarose and have stable karyotype with 45 chromosomes including two unique marker chromosomes (Morgan et al., 1991). MSU-1.0 has never been transformed to malignant by exposure to carcinogens or oncogene transfection despite intensive efforts (McCormick et al., unpublished observations). However, MSU-1.1 has been transformed by oncogene transfection or chemical carcinogen treatment and suitable selection. In many cases a progeny of selected cells form malignant tumors in athymic mice (Fry et al., 1990; Wilson et al., 1990; Yang et al., 1991).

In summary, the MSU-1 cell lineage provides us with a system in which cells are carried step wise from normal to malignant. At each step the genetic change(s) are well defined or are being defined and the resultant cells are genotypically and phenotypically stable. This cell system can now be exploited to study the effects of other carcinogens and the mechanisms of cell invasion and metastasis. Studies are being carried on by McCormick & Maher and their colleagues using the differential display technique to identify and clone the oncogene(s) and/or tumor suppressor gene(s) involved in the transformation process which are not yet understood. It

is hoped that these studies on the mechanism of malignant transformation of human fibroblasts will lead to insights which will ultimately translate into improved therapy for patients with sarcomas and perhaps other types of tumors.

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Chapter II

Malignant Transformation of Human Fibroblast Cell Strain MSU-1.1 by Ultraviolet Radiation: Correlation of Tumorigenicity with Anchorage Independence and Growth Factor Independence

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ABSTRACT

MALIGNANT TRANSFORMATION OF HUMAN FIBROBLAST CELL STRAIN MSU-1.1 BY ULTRAVIOLET RADIATION: CORRELATION OF TUMORIGENICITY WITH ANCHORAGE INDEPENDENCE AND GROWTH FACTOR INDEPENDENCE

By

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I irradiated MSU-1.1 cells,an immortal, non-tumorigenic human fibroblast cell strain with a stable, near diploid karyotype with 254nm UV radiation and selected for focus formation. MSU-1.1 is . A dose-dependent increase in the focus formation was observed. From each of eight independent irradiated cell populations, 5-6 focus-derived cell strains were selected. Several UV-induced focus derived cell stains from each irradiated cell population were tested for tumorigenicity by injecting them into athymic mice. When a cell strain from an irradiated cell population made a tumor, this cell strain and a focus-derived cell strain not able to form a tumor from the same population were selected for further analysis. Five UV-induced focus-derived cell strains

formed high grade spindle cell sarcomas in athymic mice at a high frequency and with a very short latency. These five cell strains grew well in agarose and in medium without exogenous growth factors. Two independent UV-induced focus-derived cell strains and one control focus-derived cell strain also formed fibrosarcomas in athymic mice but at a lower frequency and with a longer latency. These three cell strains did not grow well in agaros or in medium without exogenous growth factors. However two cell lines derived from these tumors (fibrosarcomas) grew well in agarose and in medium without exogenous growth factors, they grew well in agarose and in medium without exogenous growth factors. One UV-induced focus-derived cell strain formed a single fibroma after a long latency. The cells that formed the fibroma and the fibroma-derived cell line did not grow in agarose or in medium without exogenous growth factors. None of the non-tumorigenic focus-derived cell strains grew in agarose and in medium without exogenous growth factors. In summary, UV radiation can transform MSU-1.1 cells to focus formation in a dose-dependent manner and some of these focus-derived cell strains can form tumors in athymic mice. Growth in agarose and in medium without exogenous growth factors are two reliable in

vitro characteristics which correlate strongly with tumorigenicity.

INTRODUCTION

Epidemiological (Urbach, 1978 ; Scotto & Fears, 1978) and experimental studies (Ananthswamy and Pierceal,1990) indicate that ultraviolet radiation from the sun plays a causal role in the etiology and pathogenesis of human skin cancer. The most common skin cancers are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). More than 95% of the 600,000 new cases of basal and squamous cell carcinomas diagnosed each year in the USA are thought to be caused by sun exposure (Urbach, 1978; Silverberg et al., 1990). The mortality rates from such cancers are relatively low, there are about 2,500 deaths each year in United States (Scotto & Fears, 1982; Scotto et al., 1981). Recent data indicate that the depletion of the stratospheric ozone layer will increase the amount of UV radiation reaching the earth which is expected to increase the frequency of UV-induced skin cancers (Kerr & McElroy, 1993; Fears & Scotto, 1983; Lloyd, 1993).

UV-induced dipyrimidine photoproducts in DNA result in a distinctive pattern of mutations (i.e.,C-T transitions and CC-TT double base substitutions), which are often referred to as a UV signature. The presence of this signature in proto-

oncogenes and/or suppressor genes of skin tumors strongly implicates sunlight as the cause of such skin lesions (Brash et al., 1991; Vogelstein & Kinzler, 1992b; Ananthaswamy & Pierceall, 1990).

McCormick and his colleagues have developed a human fibroblast transformation system in which a normal diploid human fibroblast can be transformed to malignancy by a series of sequential selections. One cell strain in this series, MSU-1.1 has been transformed to malignancy by exposure to chemicals (Yang et al., 1992), ionizing radiations (Reinhold et al., 1996) and oncogene transfection (Hurlin et al., 1987; Hurlin et al., 1989; Fry et al., 1990; Wilson et al.,1990; Lin et al., 1995). In this study, we exposed these cells to a single dose of UV radiation, grew them for an expression period, and then selected the cells for focus formation. When assayed in the athymic mice, many of the UV-induced focus-derived cell strains were tumorigenic.

MATERIAL AND METHODS

CELL CULTURE:

Cells were cultured routinely in Eagle's minimal essential medium supplemented with L-aspartic acid (0.2 mM), L-serine (0.2mM), pyruvate (1.0 mM), 10% supplemental calf serum (SCS)

(Hyclone, Logan, UT), penicillin (100 units/ml), streptomycin (100 ug/ml) and hydrocortisone (1 ug/ml). This is referred to as culture medium. The cells were maintained in exponential growth at 37°c in a humidified incubator with 5% CO_2.

EXPOSURE TO ULTRAVIOLET RADIATION AND SELECTION OF FOCUS-FORMING CELLS:

Cells in exponential growth were plated in 150 mm-diameter culture dishes. Sixteen hours after the initial plating when the cells were well attached, the cells were exposed to ultraviolet radiation. Briefly, the cell culture medium was aspirated and the cells were washed with phosphate buffered saline, and this solution was removed. Taking care not to allow the cell monolayer to dry, we irradiated with UV (254nm) using a Mineral Light Short-Wave Lamp UVS-54 (UV Products Inc,San Gabriel CA) as previously described (Patton et al., 1984). Immediately after irradiation, cells were refed with freshly prepared culture medium. The next day, the medium was replaced to eliminate the cytotoxic effects of the dying cells. The cells were continually maintained in an exponential growth for the 7 days period following irradiation by trypsinizing and replating the cells at lower densities as required. After this expression period, from each independent

irradiated population, cells were trypsinized and pooled and counted on an electronic cell counter. 1x 10^6 cells were assayed for focus formation by plating 50,000 cell per 100 mm-diameter dish in culture medium supplemented with 0.5% SCS and 20mM Hepes (pH 7.5). The medium was renewed weekly. When distinct foci developed after 5-7 weeks, cells were isolated from representative foci. After growing for 1-2 weeks the focus-derived cells were trypsinized and plated at cloning densities to separate the non-morphologically transformed from the morphologically transformed cells. After 2 weeks, clones were identified which were made up of morphologically transformed cells. The cells from such clones were isolated and individual clonal populations were established for further analysis. The rest of the original foci were stained with methylene blue. Foci were counted and the frequency of the foci was expressed per number of cells plated.

Cytotoxity Assay

The cytotoxic effects of radiation was determined by the loss of colony forming ability. Briefly, cells in exponential growth were plated into 100 mm plastic dishes at various densities so that 25-45 colonies/dish would be observed, and irradiated 16 hours post-plating. They were refed with fresh

culture medium after irradiation and again after seven days. The colonies were stained after two weeks with crystal violet. The number of clones in the control and irradiated cells was counted and cell survival was calculated.

Growth Factor Independence Assay

The growth factor requirement of the cells was determined by a modified version of the assay of Scudiero et al.(1988). Briefly, exponentially growing MSU-1.1 cells, focus-derived cell strains, and tumor-derived cell lines were plated into 16 wells of a 96-well microtitre plate at 1000 cells/well. The cells were plated in McM medium, a modified version of MCDB110 (Ryan et al., 1987) containing 0.1 mM calcium, instead of the standard 1.0 mM concentration and 2% SCS. After 24 hours, in half of the wells the medium was replaced with 100 ul of modified McM medium lacking phenol red and containing 0.1 mM calcium which was supplemented with 10% SCS. In the rest of the wells, the medium was replaced with 100 ul of McM medium lacking phenol red and containing 0.1 mM calcium which was supplemented with the serum replacement factors designated in Ryan et al. (1987) without epidermal growth factor, referred to as Medium Without Exogenous Growth Factors (MWEGF). The cells were allowed to grow for seven

days with a medium change on the fourth day. The number of cells present was measured on days one, four and seven using 2, 3 bis(2methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino) - carbonyl]-2H-tetrazolium hydroxide)(XTT). XTT was dissolved in water at 1 mg/ml concentration and allowed to stay in 37°c water bath for approximately 20 minutes until the turbid yellow appearance was lost and the solution became clear. Just before use, phenazine methosulphate(PMS) was added to the XTT solution so that the final concentration was 0.05 mM. Taking care that the cells did not dry, medium was removed from all the wells and replaced with MWEGF in each well. A total of 50 ul of the XTT/PMS solution was added to each well already containing 200 ul of the MWEGF, using a multichannel micro well pipet, and the cells were allowed to incubate at 37°C in a 5% CO_2 incubator for four hours. This was followed by vigorous shaking at room temperature for 30 minutes. The optical density at 450 nm and 650 nm was read using an Emax™ Microplate Reader (Molecular Device, Sunnyvale, CA). All tests were carried out at least twice. The population doubling time of each cell strain was calculated from the log of OD (450-650) and plotted on log paper as a function of time (days in culture).

Anchorage Independence Assay

The cells were assayed as described by Hurlin et al. (1989) for the ability to form large sized colonies in 0.33% Sea Plaque agarose (FMC Bioproducts, Rockland, ME). The agarose plates were incubated at 37°C with 3% CO_2. MSU-1.1 cells and MSU-1.1 H-ras10 cells which were derived from MSU-1.1 by transfection of MSU-1.1 cells with a T24 H-ras oncogene were included in each assay as negative and positive controls respectively. All tests were carried out at least twice.

Tumorigenicity Assay

Absorbable gelatin sponges (Upjohn Co., Kalamazoo, MI) 1-3 cm in size were implanted subcutaneously in the subscapular and flank region of athymic BALB/c mice to serve as matrix for the cells. One week later 1 $x10^7$ exponentially growing cells in 0.2 ml of McM medium were injected directly into the sponge. Mice were monitored weekly for tumor growth and tumors were measured using a vernier caliper. In order to calculate the volume of the tumor, the formula for the volume of a sphere ($4/3\pi r^3$) was used. The radius was determined by taking half of the two diameter measurements made perpendicular to each other. The latency of the tumors was determined by plotting the tumor volume for each of the weekly measurements

against time at which it was made. The line for the tumor volume was extrapolated to cross the time axis and the point at which it crossed was called the latency of the tumor. The mice were sacrificed when the tumor reached approximately 1 cm. in diameter. A portion of the tumor was returned to culture. Since MSU-1.1 cells were generated by transfection of the v-myc gene linked to the G418-resistant gene, cells from the tumors were grown in medium containing Geneticin in order to eliminate any contaminating mouse cells.

Histopathological Analysis of Tumors

The rest of the tumor specimen was fixed in 10% Phosphate Buffered Formalin, pH 7.0, embedded in paraffin, sectioned at 4-5 um and stained with hematoxylin and eosin using the standard technique. Routine histopathological analysis was performed and tumors were graded according to the established histological criteria.

RESULTS:

UVC-INDUCED CYTOTOXICITY AND FOCUS FORMATION IN MSU-1.1 CELLS

The MSU-1.1 cells exhibit sensitivity to the cytotoxic effects of UV radiation that is not typical of that of repair proficient cells (McCormick et al., 1986)(Fig 1 A). MSU-1.1

Figure 1. Cytotoxicity and frequency of induced focus formation by UV irradiation in MSU-1.1 cells (continuous line). Cells were irradiated in exponential growth (See materials and methods for details). UV radiation resulted in a dose-dependent increase in the frquency of foci. There were 5 background foci in the untreated (control) cell population. The points are joined by the best fit line using a computer program. The dashed line shows the cytototoxicity of normal human skin fibroblast to UV radiation and is taken from a previous published study from this laboratory (McCormick et al.,1986).

Figure 1

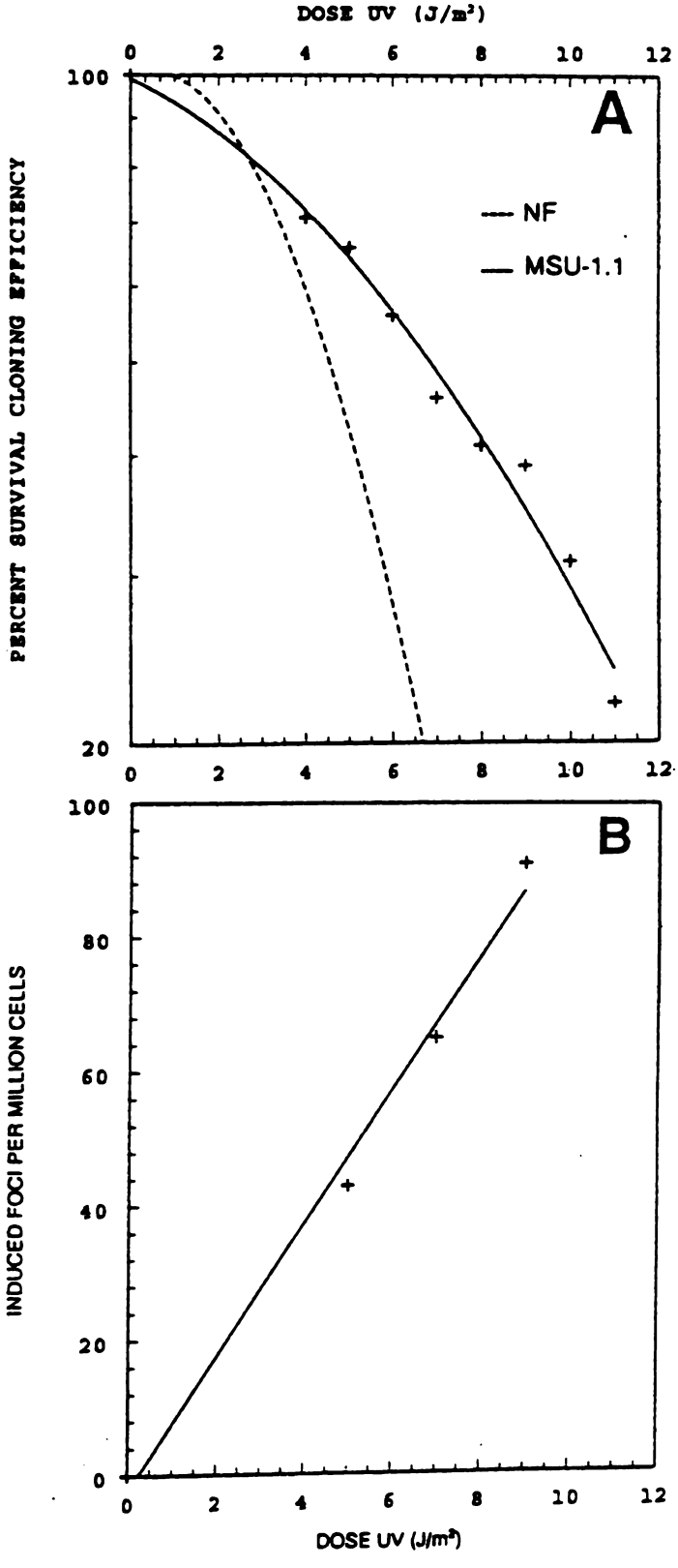

DOSE UV (J/m²)
0
2
4
6
8
10
12
100
20
A
NF
MSU-1.1
PERCENT SURVIVAL CLONING EFFICIENCY
B
80
60
40
0
INDUCED FOCI PER MILLION CELLS
DOSE UV (J/m²)

Figure 2. Phase contrast micrographs of UV-induced foci of MSU-1.1 cells.

(A): A focus of spindle cells on a background monolayer of cells. Note piling up of cells in criss-cross fashion.

(B): A focus of spindle cell on a backgound monolayer of cells. Note the absence of criss-cross pattern

Figure 2

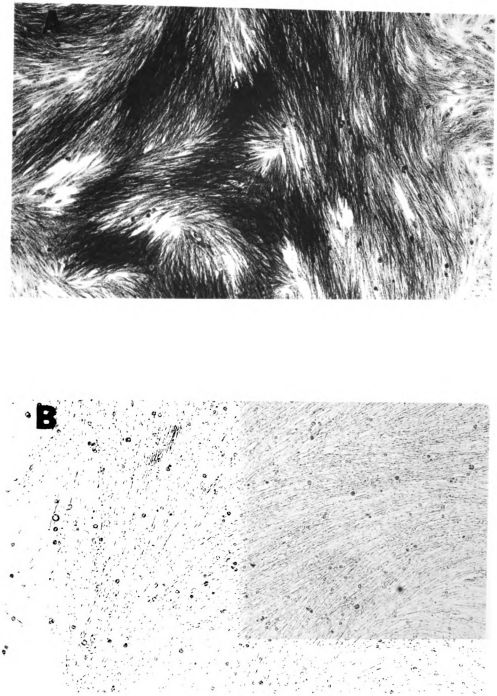


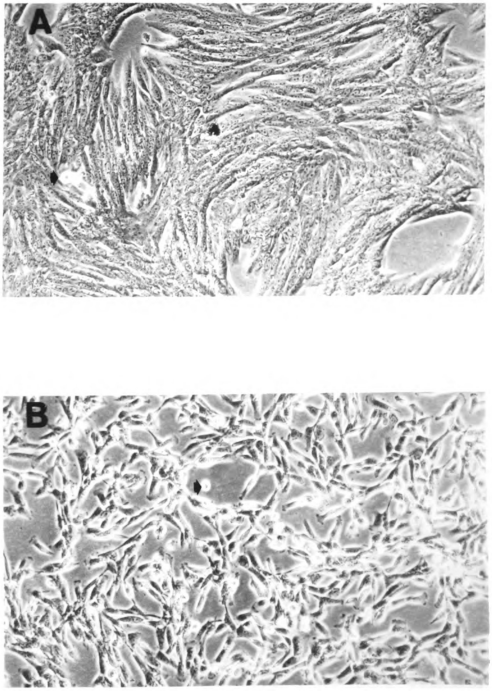
Figure 3.

Representative photomicrographs of UV-induced, focus-derived cells.

(A): Focus-derived cell strain MSU-1.1-UVC2-B5 exhibited a spindle shape. This cell strain produced high grade spindle cell sarcomas in athymic mice.

(B): Focus-derived cell strain MSU-1.1-UVC2-B11a exhibited a spindle shape and grew in an open pattern. This cell strain produced high grade spindle cell sarcomas in athymic mice.

Figure 3



MSU-1.1 cells were exposed to different doses of ultraviolet radiation and then assayed for focus formation, as detailed in the materials and methods. There was a dose dependent increase in the foci (Fig 1B). The foci stained more darkly than the monolayer and were refractile when viewed with a phase contrast microscope before staining. Fig 2(A,B) shows the phase contrast micrograph of foci. Phase contrast microscopic examination showed that most foci consists of spindle-shaped cells which form a dense criss-cross, multi-layers. In a few foci the cells appeared to be more plumb and exhibited increased piling up and numerous mitotic figures (Fig 2A). In culture, the UV-induced focus-derived cells mainly have a spindle shape(Fig 3A and 3B).

SELECTION OF INDEPENDENT UV-INDUCED FOCUS-DERIVED CELL STRAINS

Eight independent cell populations were irradiated by UV and placed in the focus assay. At the end of the each focus assay, cells were isolated from 5-6 foci, cloned twice to isolate pure population of morphologically transformed cells, expanded and injected into athymic mice for tumorigenicity. From each independently-irradiated cell population, the cell strain which formed tumors in the athymic

mice first was selected for further study. A non-tumorigenic cell strain derived from a focus that occurred in the same cell population was also selected for further study. In the non-irradiated (control) cell populations, the number of background foci was very low(5-6 per million cells). We selected one control focus-derived cell strain that was tumorigenic and one that was not tumorigenic for further study.

Tumorigenicity Assay

Eight independent focus-derived cell strains from independently irradiated cell populations and one control (untreated) focus-derived cell strains formed tumors in mice (Table 1). The tumorigenic cell strains fall into categories according to the frequency and latency of the tumors. Five focus-derived cell strains (1.1-UVC2-B5, 1.1-UVC2-C16, 1.1-UVC1-B11a, 1.1-UVC2-B11b and 1.1-UVC2-C1a)formed sarcomas with a short latency (2-6 weeks) and high frequency (87-100%). All these tumors were high grade sarcomas. See Fig 4a and 4b for typical microscopic appearance. Frequent mitosis and an occasional tripolar mitotic figure was seen in these tumors

mice first was selected for further study. A non-tumorigenic cell strain derived from a focus [illegible] the same cell population was also selected [illegible] the non-irradiated (control) [illegible] background foci was very [illegible] selected one control [illegible] tumorigenic and one [illegible] study.

Tumorigenicity Assay

Eight in[illegible] independently irradi[illegible] (untreated) focus-[illegible] (Table 1). The tumori[illegible] according to the frequen[illegible] Five focus-derived cell strains [illegible] C16, 1.1-UVC1-B11a, 1.1-UVC2-B11b and 1.1-UVC2-[illegible] formed sarcomas with a short latency (2-6 weeks) and high frequency (87-100%). All these tumors were high grade sarcomas. See Fig 4a and 4b for typical microscopic appearance. Frequent mitosis and an occasional tripolar mitotic figure was seen in these tumors

Table 1: Anchorage Independence, Growth Factor Independence and Tumorigenicity of UVC-Induced and Control Focus-Derived Cell Strains

MSU-1.1 DERIVED CELL STRAINS	UV DOSE (JOULES/M²)	ABILITY TO FORM AGAR COLONIES[A]	GROWTH FACTOR INDEPENDENCE[B]	TUMORIGENICITY FREQUENCY[C]	TUMORIGENICITY LATENCY[D]
MSU-1.1	0	+	+	0/50	NA
MSU-1.1-UVC2-CONT1	0	+	+	0/4	NA
MSU-1.1-UVC2-CONT2	0	+	++	3/4	10-12
MSU-1.1-UVC2-B5	9	+++	++	6/6	3-5
MSU-1.1-UVC2-C16	9	+++	++	5/5	2-4
MSU-1.1-UVC1-B11a	9	+++	++	4/4	4-6
MSU-1.1-UVC2-B11b	12	+++	++	7/8	3-6
MSU-1.1-UVC2-C1a	12	+++	++	4/4	2-4
MSU-1.1-UVC2-A16b	9	+	+	3/4	12-18
MSU-1.1-UVC2-A17	12	+	+	2/8	18-20
MSU-1.1-UVC1-A18b	12	+	+	1/8	20
MSU-1.1-UVC2-B19	9	+	+	0/4	NA
MSU-1.1UVC2-C1b	9	+	+	0/4	NA
MSU-1.1-UVC1-B15	9	+	+	0/4	NA
MSU-1.1-UVC2-B4	12	+	+	0/4	NA
MSU-1.1-UVC2-C20	12	+	+	0/4	NA
MSU-1.1-UVC2-A14	12	+	+	0/4	NA
MSU-1.1-UVC2-A15	9	+	+	0/4	NA
MSU-1.1-UVC1-A3	12	+	+	0/4	NA

A) +, frequency of agar colonies <0.01%,size>60um; ++, frequency of agar colonies 5-15%, size >100um

+++, frequency of agar colonies 5-15%, size >160um

B) +, moderate growth in MWEGF, like normal human diploid fibroblasts in medium supplemented with 5% serum.

++, rapid growth in MWEGF, like normal human diploid fibroblast in medium supplemented with 10% serum.

C) Frequency indicates the number of tumors per number of sites injected in the athymic mice.

D) Refer to the description of latency in Materials and Methods

NA= Not applicable

Table 1: Anderegg Independence, Growth Factor Independence and Tumorigenicity of UVC-Induced and Control Farm Human Cell Lines

Figure 4

Histology of tumors produced by subcutaneous injection of UV-induced-focus-derived cell strains.

(A): Representative section of a poorly differentiated spindle cell sarcoma produced by cell strain MSU-1.1-UVC2- B5, which produced tumors at all injection sites with a short latency. Note the compact cellular architecture with scant intracellular matrix and multiple mitotic figures (6-8 per high power field).

(B): Representative section of a high grade sarcoma with pleomorphic cells produced by cell strain MSU-1.1 UVC2-C16 , which produced tumors at all injection sites with a short latency.

Figure 4

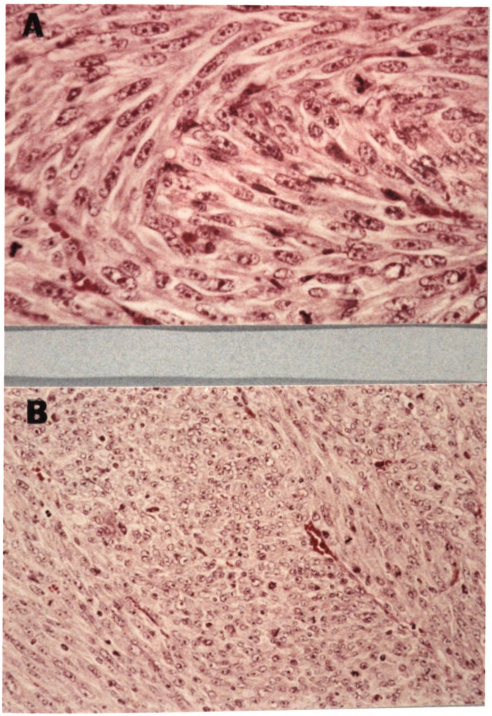
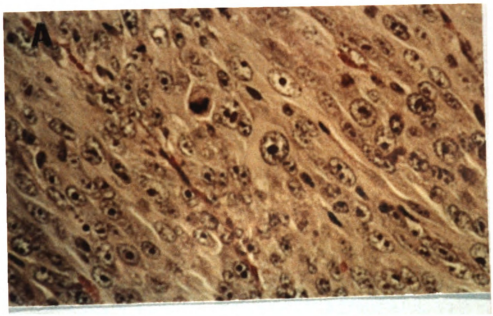


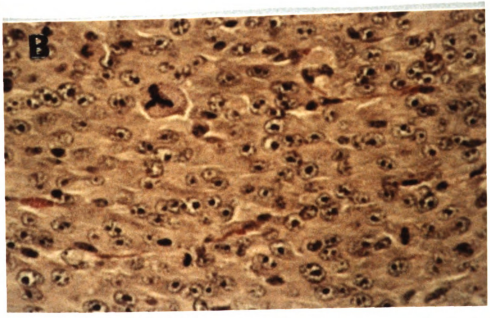
Fig 4 (cont'd)

C. Representative section of a high grade spindle cell sarcoma with frequent mitosis and scant intercellular matrix produced by cell strain MSU-1.1- UVC2-11b, which produced tumors at all injection sites with a short latency. Note the classical tripolar mitotic figure(see arrow). Most of the cells are spindle shaped with a high degree of anaplasia.

D. Representative section of a high grade sarcoma with frequent abnormal mitotic figures (6-8 per high power field) produced by cell strain MSU-1.1-UVC2- C1a, which produced tumors at all injection sites with a short latency

Figure 4, cont'd





(Fig 4C and 4D). Two focus-derived cell strains (1.1-UVC2-A16b and 1.1-UVC2.A17) formed tumors at a lower(25-75%) frequency with a relatively long latency (12-20 weeks). Another focus-derived cell strain (1.1-UVC2.A18b) formed a single fibroma after a long latency (20 weeks). One of the control focus-derived cell strains (1.1-UVC2-CONT2) formed tumors at a 75% of the injection sites and with a moderate latency (10-12 weeks). None of the rest of focus-derived cell strains formed tumors in the athymic mice . Therefore we selected eight non-tumorigenic UV-induced-focus-derived cell strains, one from each independent UV irradiated cell population, for further characterization.

GROWTH FACTOR INDEPENDENCE OF UV-INDUCED FOCUS DERIVED CELL STRAINS

Previous studies in this laboratory showed that oncogene and an active derivative of benzo(a)pyerene transformed MSU-1.1 cells into cells that are highly tumorigenic and proliferate rapidly in medium lacking exogenous growth factors. To examine whether UV-transformed focus-derived cells have this property, all the cell strains were tested for growth in medium without exogenous growth factors. MSU-1.1 cells, which grow only moderately well under these conditions,

were included as a negative control and MSU-1.1 cells transformed by the H-ras oncogene (MSU-1.1-H-Ras10) were used as a positive control. This cell strain is known to be growth factor independent (J. J. McCormick, unpublished studies). The results of the growth factor independence assays are summarized in Table 1. Five cell strains(1.1-UVC2-B5, 1.1-UVC2-C16, 1.1-UVC1-B11a, 1.1-UVC2-B11b and 1.1-UVC2-C1a) were completely growth factor independent(i.e., GFI++). These cell strains replicated as rapidly in MWEGF as the parental MSU-1.1 cells replicate in medium supplemented with 10% SCS. These five cell strains are the ones which formed sarcomas at a high frequency and short latency. The UV-induced focus-derived cell strains which formed tumors at a lower frequency with a relatively long latency(1.1-UVC2-A16b, 1.1-UVC2-A17 and 1.1-UVC2-control2) and a UVC-induced focus-derived cell strain which formed a single fibroma (1.1-UVC1-B18b) grew like the parental MSU-1.1 cells in medium without exogenous growth factors (i.e., GFI +). The other focus-derived cell strains also grew like MSU-1.1 cells in the medium without exogenous growth factors.

ANCHORAGE INDEPENDENCE OF UV-INDUCED FOCUS-DERIVED CELL STRAINS

UV-induced focus-derived cell strains were tested for their ability to form large size colonies in agarose. The results of the agarose assays are shown in Table 1. The five UV-induced cell strains (1.1-UVC2-B5, 1.1-UVC2-C16, 1.1-UVC1-B11a, 1.1-UVC2-B11b, and UVC2-C1a) which formed tumors at a high frequency and with a short latency, formed large size colonies at higher frequencies (i.e.,AI+++)(Fig 5A and 5B). The control focus-derived cell strain (1.1-UVC2-control2)grew moderately well in agarose (i.e.,AI++)(Fig 6a). All of the others focus-derived cell strains grew like the parental MSU-1.1 cells in agarose (i.e.,AI+)(Fig 6B).

GROWTH IN AGAROSE AND IN MEDIUM WITHOUT EXOGENOUS GROWTH FACTORS OF SOME OF THE TUMOR-DERIVED CELL LINES.

The five focus derived cell strains which grow well in agarose (AI+++) and in medium without exogenous growth factors (GFI++) formed high grade sarcomas in athymic mice at a relatively high frequency and with a short latency. However, MSU-1.1-UVC2-A16b, MSU-1.1-UVC2-A17 and MSU-1.1-UVC-control2, which did not grow well in agarose or in medium without exogenous growth factors, also formed tumors in athymic mice

UV-induced focus-derived cell strains were tested for their ability to form large size colonies in agarose. The results of the agarose assays are shown in [illegible]. The three UV-induced cell strains (1.1-UVC2-[illegible] B11a, 1.1-UVC2-B11b, and UVC[illegible] high frequency and with [illegible] colonies at higher freq[illegible] The control focus-der[illegible] moderately well in ag[illegible] others focus-derived [illegible] 1.1 cells in agarose [illegible]

GROWTH IN AGAROSE AND [illegible] FACTORS OF SOME OF THE [illegible]

The five focus-der[illegible] well in agarose (AI+++) and in medium [illegible] growth factors (GFI++) formed high grade [illegible] in athymic mice at a relatively high frequency and with a short latency. However, MSU-1.1-UVC2-A15b, MSU-1.1-UVC2-A17 and MSU-1.1-UVC-control2, which did not grow well in agarose or in medium without exogenous growth factors, also formed tumors in athymic mice

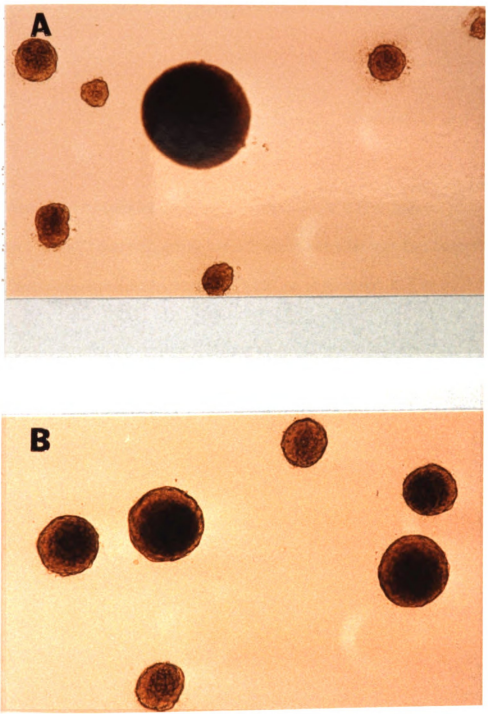
Figure 5.

Colony formation by UV-induced focus-derived cell strains in 0.33% agarose.

A. Cell strain MSU-1.1-UVC2-B5, which produced high grade sarcomas with a short latency at all injection sites, formed large size colonies in agarose (200-300 μm diameter) in 3 weeks.

B. Cell strain MSU-1.1-UVC1-B11a which also produced a high grade sarcomas with a short latency at 7 of 8 injection sites , formed large size colonies in agarose (200-300μm in diameter) in 3 weeks.

Figure 5



but with a relatively long latency (Table 1). Previous studies in this laboratory showed that if focus-derived MSU-1.1 cell strains which did not grow well in agarose and in medium without exogenous growth factors formed tumors, the tumors had a long latency. In contrast, the cell lines derived from these tumors grew well in agarose and in medium without exogenous growth factors (Yang et al., 1992). In view of these findings, tumor-derived cell lines 1.1-UV2.A16b/T and 1.1-UV2-control 2/T derived from high grade sarcomas were tested for growth in agarose and medium without exogenous growth factors. MSU-1.1-UVC2-A17 was lost in a freezer accident and could not be tested. These tumor-derived cell lines formed large colonies at a high frequency (i.e., AI+++), and they grew rapidly in MWEGF, like normal human fibroblasts grow in medium supplemented with 10% serum (i.e., GFI++), whereas the original focus-derived cells did not do so (Table 2). However, the fibroma-derived cell line (1.1-UV1-A18b/T) did not grow well(i.e., AI+/GFI+). Indeed, they grew just like the parental MSU-1.1 cells (Table 2).

Table 2: Anchorage Independence, Growth Factor Independence and Tumorigenicity of Tumor-Derived Cell Lines and Corresponding Focus-Derived Cell Strains.[a]

CELL STRAIN/CELL LINE	FOCUS/TUMOR	ANCHORAGE INDEPENDENCE[A]	GROWTH FACTOR INDEPENDENCE[B]	TUMOR Frequency[C]	TUMOR Latency[D]
MSU-1.1-UVC2-A16b	Focus-derived	+	+	3/4	12-18
MSU-1.1-UVC2-A16b/T	Tumor-derived	+++	++	4/4	3-5
MSU-1.1-UVC2-CONTROL2	Focus-derived	++	+	3/4	10-12
MSU-1.1-UVC2-CONTROL2/T	Tumor-derived	+++	++	4/4	3-4
MSU-1.1-UVC1-A18B	Focus-derived	+	+	1/8	20
MSU-1.1-UVC2-A18B/T	Tumor-derived	+	+	0/4	NA

a: See Table 1 for legend and information concerning these focus-derived cell strains.

Table 2. Anchorage Independence, Growth Factor Independence, and Tumorigenicity of Tumor-Derived Cell Lines and Primary Tumor-Derived Cell Strains.*

CELL STRAIN/CELL LINE	TUMOR ORIGIN	[illegible]	[illegible]
MDH-LEC3-A8b	Benign skeletal		
MDH-LEC3-A9M7	Tumor-derived		
MDH-LEC3-C10M3A1	Tumor-derived		
MDH-LEC3-C10M7	Tumor-derived		
MDH-LEC3-C10M8	Tumor-derived		
MDH-LEC3-C10B	Benign skeletal		
MDH-LEC3-E10M7	Tumor-derived		

* See Table 1 for legend and references.

DISCUSSION:

These experiments demonstrate that a single dose of UV radiation is able to induce MSU-1.1 cells to form foci in a dose- dependent manner and that some of the focus-derived cells are tumorigenic. The frequency of focus formation increases linearly with UV dose(Fig 1) and at 25-30% is similar to the number of HPRT mutations induced in normal skin fibroblasts by this radiation (100-120 per million)(Maher et al., 1982) and by a reeactive derivative of benzo(a)pyrene (Yang et al., 1991; Yang et al., 1992) suggesting that focus formation may be caused by a mutation.

Our study is similar to two other studies from this laboratory using MSU-1.1 cells. Reinhold et al. (1996) reported that ionizing radiation was able to cause focus formation in MSU-1.1 cells and that the progeny cells of some foci are highly tumorigenic. Yang et al.(1992) reported that, an active derivative of benzo(a)pyrene was able to induce foci. In this study, cell strains derived from three foci formed a high frequency of large colonies in agarose and grew without exogenous growth factors. These cells formed tumors that reached 6 mm diameter in two-to-three weeks in all animals. One cell strain (2C1) that did not form large

colonies in agarose and did not grow without exogenous growth factors formed tumors in three of four mice after two and a half months. However, the cells derived from these tumors formed large colonies in agarose at a high frequency and grew rapidly in medium without exogenous growth factors just as the other three tumorigenic strains had. The interpretation was that a variant cell(s) with these characteristics arose spontaneously in the 2C1 cell population and that these variant cells formed the tumors.

In the present experiment, the five UV-induced focus-derived cell strains that formed large colonies in agarose at a high frequency and grew rapidly without exogenous growth factors were tumorigenic in 87-100% of the mice, and the tumors formed were of the shortest latency (2-6 weeks)(Table 1). Nine focus-derived cell strains that did not exhibit growth without exogenous growth factors and did not form colonies in agarose were not tumorigenic. Three cell strains that did not exhibit growth in medium without exogenous growth factors and which exhibited little or no ability to form colonies in agarose were found to give rise to tumors at 25-75% of the injection sites. These tumors were of relatively longer latency (12-20 weeks). When cells derived from these

tumors were tested for these characteristics, they were found to grow without exogenous growth factors, to form large colonies in agarose, and to form tumors with a very short latency(3-5 weeks) (Table 2). These cell strains appear to be like the 2C1-derived cell strain described above, from the research of Yang et al.(1992). Our interpretation is that variant cells that had these characteristics arose spontaneously in the cell populations after they were isolated from the foci and it was the expression of these characteristics that converted them to tumorigenic cells. One cell stain which was derived from a fibroma did not have these characteristics and may be a special case.

The results we have obtained are similar to that of Smith et al. (1993) who studied the transformation of an infinite life span mouse fibroblast cell line (C3H10T1/2) by various chemical carcinogens. They found that 81% of the focus-derived cell populations that formed large size clonies in agarose were tumorigenic. In the present study and that of Yang et al., 1992, 100% of the focus-derived MSU-1.1 cells that grew rapidly without exogenous growth factors and formed large colonies in agarose at a high frequency were tumorigenic.

Our study supports the hypothesis of Smets'(1982), who suggested that transformation in vitro occurs by distinct steps. At each step, cells acquire independent, qualitatively distinct properties like colony formation in agarose, growth in medium without exogenous growth factors, etc. In our study, MSU-1.1 cells showed a somewhat greater senstivity to the cytotoxic effects of UV 254 when compared with normal skin fibroblasts(McCormick et al., 1986). The reasons for this are not known but the cause cannot be the loss of expression of wild type p53 protein as found by Ford and Hanawalt (1995) since MSU-1.1 cells express wild type p53 protein (J.J.McCormick, unpublished studies).

In summary, our data show that exposure of MSU-1.1 cells to UV radiation causes increase in the frquency of foci in a dose-dependent manner. The progeny of cells from some of these UV-induced foci are tumorigenic, but not cells from every focus. We conclude that co-expression of three in vitro biological characteristics i.e., the ability to form foci, the ability to form large size colonies in agarose at high frequency and the ability to grow in medium without exogenous growth factors correlates with the ability of the cell strains to form tumors in athymic mice.

Our study supports the hypothesis [illegible] (19[illegible]), who suggested that transformation [illegible] distinct steps. At each step, cells acquire [illegible] distinct properties like [illegible] in medium without exogenous [illegible] study, MSU-1.2 cells showed [illegible] the cytotoxic effects of [illegible] fibroblasts (McCormick [illegible] not known but the [illegible] wild type p53 protein [illegible] since MSU-1.1 [illegible] (J.J.McCormick, unpub[illegible]

In summary, [illegible] cells to UV radiation [illegible] in a dose-dependent manner. [illegible] some of these UV-induced foci are tumorigenic, but not cells from every focus. We conclude that co-expression of three in vitro biological characteristics i.e., the ability to form foci, the ability to form large size colonies in agarose at high frequency and the ability to grow in medium without exogenous growth factors correlates with the ability of the cell strains to form tumors in athymic mice.

ACKNOWLEDGMENTS

We wish to thank Suzanne K Kohler, Lonnie Milam and other members of Carcinogenesis Laboratory for their help and assistance. This research was supported in part by DHHS grant CA 56796 from the NCI.

ABBREVIATIONS:

AI, anchorage independence; GFI, growth factor independence UV, ultraviolet; MWEGF, medium without exogenous growth factors

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Figure 5.

Colony formation by UV-induced focus-derived cell strains in 0.33% agarose.

A. Cell strain MSU-1.1-UVC2-B5, which produced high grade sarcomas with a short latency at all injection sites, formed large size colonies in agarose (200-300 μm diameter) in 3 weeks.

B. Cell strain MSU-1.1-UVC1-B11a which also produced a high grade sarcomas with a short latency at 7 of 8 injection sites , formed large size colonies in agarose (200-300μm in diameter) in 3 weeks.

Figure 5

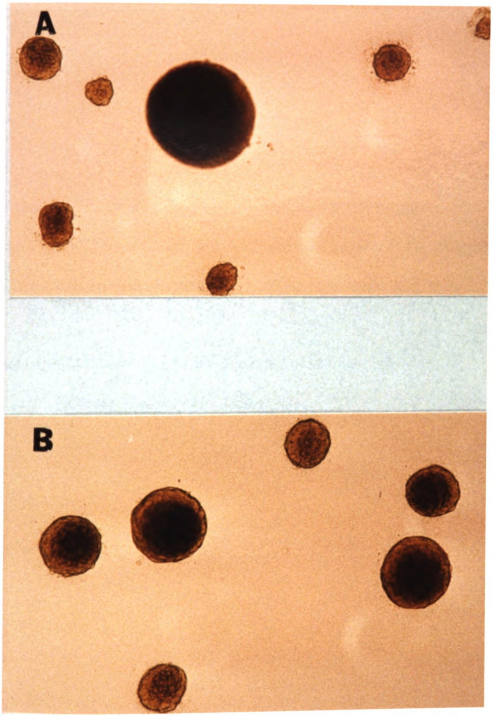


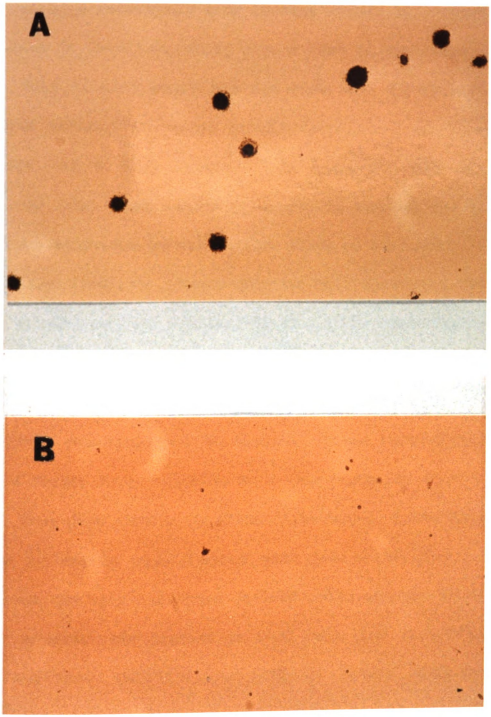
Figure 6.

Colony formation by UV-induced, focus-derived cell strains in 0.33% agarose.

A. Cell strain 1.1-UVC2-control2, which formed tumors with relatively long latency, formed moderate size agarose colonies (60-100 μm in diameter) in 3 weeks.

B. Cell strain MSU-1.1-UVC2-B15, which was not tumorigenic, formed small agarose colonies (40-60 μm diameter) in 3 weeks.

Figure 6



but with a relatively long latency (Table 1). Previous studies in this laboratory showed that if focus-derived MSU-1.1 cell strains which did not grow well in agarose and in medium without exogenous growth factors formed tumors, the tumors had a long latency. In contrast, the cell lines derived from these tumors grew well in agarose and in medium without exogenous growth factors (Yang et al., 1992). In view of these findings, tumor-derived cell lines 1.1-UV2.A16b/T and 1.1-UV2-control 2/T derived from high grade sarcomas were tested for growth in agarose and medium without exogenous growth factors. MSU-1.1-UVC2-A17 was lost in a freezer accident and could not be tested. These tumor-derived cell lines formed large colonies at a high frequency (i.e., AI+++), and they grew rapidly in MWEGF, like normal human fibroblasts grow in medium supplemented with 10% serum (i.e., GFI++), whereas the original focus-derived cells did not do so (Table 2). However, the fibroma-derived cell line (1.1-UV1-A18b/T) did not grow well(i.e., AI+/GFI+). Indeed, they grew just like the parental MSU-1.1 cells (Table 2).

Table 2: Anchorage Independence, Growth Factor Independence and Tumorigenicity of Tumor-Derived Cell Lines and Corresponding Focus-Derived Cell Strains.[a]

CELL STRAIN/CELL LINE	FOCUS/TUMOR	ANCHORAGE INDEPENDENCE[A]	GROWTH FACTOR INDEPENDENCE[B]	TUMOR Frequency[C]	TUMOR Latency[D]
MSU-1.1-UVC2-A16b	Focus-derived	+	+	3/4	12-18
MSU-1.1-UVC2-A16b/T	Tumor-derived	+++	++	4/4	3-5
MSU-1.1-UVC2-CONTROL2	Focus-derived	++	+	3/4	10-12
MSU-1.1-UVC2-CONTROL2/T	Tumor-derived	+++	++	4/4	3-4
MSU-1.1-UVC1-A18B	Focus-derived	+	+	1/8	20
MSU-1.1-UVC2-A18B/T	Tumor-derived	+	+	0/4	NA

a: See Table 1 for legend and information concerning these focus-derived cell strains.

Table 2: Anchorage Independence, Growth Factor Independence and Tumorigenicity of Tumor-Derived Cell Lines and "Premorbid Pre-Tumor Cell Strains."

CELL STRAIN/CELL LINE	POSITIVE/TOTAL		
MDU-1-EVC5-A189	Pre-tumor		
MDU-1-EVC5-A9MT	Tumor-derived		
MDU-1-EVC5-CONTROL1	Pre-tumor		
MDU-1-EVC5-CONTROL2T	Tumor-derived		
MDU-1-EVC5-A189	Pre-tumor		
MDU-1-EVC5-A189T	Tumor-derived		

* See Table 1 for legend and footnotes.

DISCUSSION:

These experiments demonstrate that a single dose of UV radiation is able to induce MSU-1.1 cells to form foci in a dose- dependent manner and that some of the focus-derived cells are tumorigenic. The frequency of focus formation increases linearly with UV dose(Fig 1) and at 25-30% is similar to the number of HPRT mutations induced in normal skin fibroblasts by this radiation (100-120 per million)(Maher et al., 1982) and by a reeactive derivative of benzo(a)pyrene (Yang et al., 1991; Yang et al., 1992) suggesting that focus formation may be caused by a mutation.

Our study is similar to two other studies from this laboratory using MSU-1.1 cells. Reinhold et al. (1996) reported that ionizing radiation was able to cause focus formation in MSU-1.1 cells and that the progeny cells of some foci are highly tumorigenic. Yang et al.(1992) reported that, an active derivative of benzo(a)pyrene was able to induce foci. In this study, cell strains derived from three foci formed a high frequency of large colonies in agarose and grew without exogenous growth factors. These cells formed tumors that reached 6 mm diameter in two-to-three weeks in all animals. One cell strain (2C1) that did not form large

DISCUSSION:

colonies in agarose and did not grow without exogenous growth factors formed tumors in three of four mice after two and a half months. However, the cells derived from these tumors formed large colonies in agarose at a high frequency and grew rapidly in medium without exogenous growth factors just as the other three tumorigenic strains had. The interpretation was that a variant cell(s) with these characteristics arose spontaneously in the 2C1 cell population and that these variant cells formed the tumors.

In the present experiment, the five UV-induced focus-derived cell strains that formed large colonies in agarose at a high frequency and grew rapidly without exogenous growth factors were tumorigenic in 87-100% of the mice, and the tumors formed were of the shortest latency (2-6 weeks)(Table 1). Nine focus-derived cell strains that did not exhibit growth without exogenous growth factors and did not form colonies in agarose were not tumorigenic. Three cell strains that did not exhibit growth in medium without exogenous growth factors and which exhibited little or no ability to form colonies in agarose were found to give rise to tumors at 25-75% of the injection sites. These tumors were of relatively longer latency (12-20 weeks). When cells derived from these

tumors were tested for these characteristics, they were found to grow without exogenous growth factors, to form large colonies in agarose, and to form tumors with a very short latency(3-5 weeks) (Table 2). These cell strains appear to be like the 2C1-derived cell strain described above, from the research of Yang et al.(1992). Our interpretation is that variant cells that had these characteristics arose spontaneously in the cell populations after they were isolated from the foci and it was the expression of these characteristics that converted them to tumorigenic cells. One cell stain which was derived from a fibroma did not have these characteristics and may be a special case.

The results we have obtained are similar to that of Smith et al. (1993) who studied the transformation of an infinite life span mouse fibroblast cell line (C3H10T1/2) by various chemical carcinogens. They found that 81% of the focus-derived cell populations that formed large size clonies in agarose were tumorigenic. In the present study and that of Yang et al., 1992, 100% of the focus-derived MSU-1.1 cells that grew rapidly without exogenous growth factors and formed large colonies in agarose at a high frequency were tumorigenic.

Our study supports the hypothesis of Smets'(1982), who suggested that transformation in vitro occurs by distinct steps. At each step, cells acquire independent, qualitatively distinct properties like colony formation in agarose, growth in medium without exogenous growth factors, etc. In our study, MSU-1.1 cells showed a somewhat greater senstivity to the cytotoxic effects of UV 254 when compared with normal skin fibroblasts(McCormick et al., 1986). The reasons for this are not known but the cause cannot be the loss of expression of wild type p53 protein as found by Ford and Hanawalt (1995) since MSU-1.1 cells express wild type p53 protein (J.J.McCormick, unpublished studies).

In summary, our data show that exposure of MSU-1.1 cells to UV radiation causes increase in the frquency of foci in a dose-dependent manner. The progeny of cells from some of these UV-induced foci are tumorigenic, but not cells from every focus. We conclude that co-expression of three in vitro biological characteristics i.e., the ability to form foci, the ability to form large size colonies in agarose at high frequency and the ability to grow in medium without exogenous growth factors correlates with the ability of the cell strains to form tumors in athymic mice.

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ABBREVIATIONS:

AI, anchorage independence; GFI, growth factor independence UV, ultraviolet; MWEGF, medium without exogenous growth factors

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