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THE TUMOR-FORMING CAPACITY OF HUMAN FIBROBLASTS MALIGNANTLY TRANSFORMED *IN VITRO* AND *IN VIVO*

By

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ABSTRACT

THE TUMOR-FORMING CAPACITY OF HUMAN FIBROBLASTS MALIGNANTLY TRANSFORMED *IN VITRO* AND *IN VIVO*

By

John Edward Dillberger

To study the role of ras oncogenes in human tumorigenesis and tumor pathology, I examined tumors produced in athymic nude mice by N-ras-, H-ras-, and K-ras-transformed human cell strains, by human sarcoma-derived cell lines, and by a spontaneously-transformed human cell line. I also conducted experimental metastasis studies using ras-transformed cell strains. Injected subcutaneously, the ras-transformed cell strains made progressively-growing, invasive sarcomas that were classified by microscopic features as myxoid sarcomas, spindle cell sarcomas (with and without whorls), round cell sarcomas, pleomorphic sarcomas (with mononuclear or multinuclear giant cells) and malignant mesenchymomas. One H-ras-transformed cell strain made S-100-positive sarcomas, and two N-ras-transformed cell strains made desmin-positive sarcomas. The transfected ras oncogene and the recipient cell line both influenced the type of tumor produced by a given cell strain. The sarcoma-derived cell lines and spontaneously-transformed cell line also made sarcomas of several morphologic types, some of which were S-100- and desmin-positive. The ras-transformed cell strains did not metastasize spontaneously from primary tumors in the subcutis but were capable of experimental metastasis via the bloodstream or by implantation. Metastatic ability was influenced by the transfected ras oncogene and the recipient cell line.

This work is dedicated to the memory of Mr. Brian Lanier, whose death in 1987 reminded me that more is at stake in cancer research than the satisfaction of my own intellectual curiosity.

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Beyond these direct contributions I wish to express my gratitude to three others who had a profound impact on my studies. First, I thank Dr. Norman Altman for providing the solid grounding in comparative pathology that served me so well in this research. Second, I thank Dr. Walter L. Brown, whose unflagging curiosity and lifelong love of learning have been a constant source of inspiration. And finally, I thank my wife, CiGi, whose love, support, encouragement, enthusiasm, and strength have never wavered, and who always seemed to know which of them I needed most at any moment.

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BPDE = (±)-7B,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene

BSA = bovine serum albumin

DEN = diethylnitrosamine

DMBA = dimethylbenzanthracene

DNA = deoxyribonucleic acid

EBV = Epstein-Barr virus

EDTA = ethylenediaminetetraacetic acid

ENU = ethylnitrosourea

FCS = fetal calf serum

GDP = guanosine diphosphate

GTP = guanosine triphosphate

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

MNU = N-nitroso-N-methylurea

SV40 = simian virus 40

4-NQO = 4-nitroquinoline 1-oxide

5-AzaC = 5-azacytidine

INTRODUCTION

Over the past 200 years, pathologists have accumulated a vast body of information on human tumor pathology. Much of that information consists of correlations between the microscopic features of human tumors and the behavior of those tumors in vivo. These correlations allow pathologists to predict the behavior of a tumor (offer a prognosis) by its microscopic features (diagnosis). Recently, pathologists have begun to investigate the molecular basis for the microscopic features on which they rely (Cline, 1989). Techniques such as immunohistochemical stains, immunoprecipitation, and Western blotting have enabled investigators to detect and measure specific proteins within tumor cells, while Southern analysis, Northern analysis, and in situ hybridization have permitted them to detect specific genes and mRNA transcripts within cells.

At the same time such techniques were becoming available to pathologists, in vitro studies on rodent cell transformation revealed that specific genes, called oncogenes, were involved in carcinogenesis (Cooper, 1982; Weinberg, 1982; Varmus, 1984; Bishop, 1985 and 1987; Nishimura and Sekiya, 1987). Oncogenes turned out to be altered or inappropriately expressed forms of normal cellular genes. The first oncogene identified was a ras oncogene from a human bladder carcinoma. Using the techniques of molecular analysis, pathologists and other cancer researchers have analyzed many human tumors for oncogenes. The oncogenes they have found most often belong to the ras family (Shih and Weeks, 1984; Barbacid, 1987).

The widespread occurrence of ras oncogenes in human tumors raises the question, is there a relationship between ras oncogenes and the microscopic features of human

tumors? Most studies in which rodent cells have been transformed by ras transfection or infection with a ras-containing retrovirus have not addressed the question because they have focused on the role of ras in the transformation process itself, and the investigators have viewed tumor formation by the ras-transformed cells as an endpoint. The few investigators who have examined tumors produced by ras-transformed rodent cells seldom have attempted to correlate the histopathologic features of the tumors with the method of transformation. Attempts to do so have been complicated by the relatively high rate of spontaneous transformation in many rodent cell systems.

An experimental system for investigating the role of ras oncogenes in human tumor pathology recently became available in the Carcinogenesis Laboratory at Michigan State University with the successful in vitro transformation of human fibroblasts by transfected ras oncogenes (McCormick et al., 1989). A series of tumorigenic ras-transformed human cell strains, created by transfection of infinite life span human fibroblasts with an H-ras (Hurlin et al., 1989), N-ras (Wilson et al., 1989b), or K-ras (Fry et al., 1989) oncogene, was on hand when I joined the laboratory in 1986. The studies described in this dissertation utilitzed those ras-transformed cell strains, as well as, two spontaneously-transformed cell strains (L55I-3T and VIP:F-T), and a series of sarcoma-derived cell lines. This dissertation begins by reviewing current concepts of carcinogenesis and the role of oncogenes, with a detailed discussion of ras oncogenes emphasizing the relationship between ras oncogenes and human tumor pathology. The studies I conducted on the tumor-forming capacity of various cell lines and strains are described in the next three sections, which are prepared as manuscripts to be submitted for publication in Laboratory Investigation (Chapters VI and VIII) and The Journal of the National Cancer Institute (Chapter VII). These chapters are organized in formats that comply with the requirements of those journals. The Appendix

describes studies in which I attempted unsuccessfully to induce human fibroblasts to acquire an infinite life span in vitro by treating them with ENU or 5-AzaC.

BACKGROUND AND LITERATURE REVIEW

Oncogenes and Carcinogenesis

Most evidence to date indicates that carcinogenesis is a multi-step process. This conclusion is supported by epidemiologic studies of human cancer (Doll, 1978), experimental studies on tumor induction in animals (Boutwell, 1974; Farber and Sarma, 1987), and in vitro studies on cell transformation (Barrett and Ts'o, 1978; Thomassen et al., 1985). Decades of research also suggest that much human cancer is the result of environmental mutagens or carcinogens, both natural and synthetic, to which all of us are exposed in the food we eat, the water we drink, or the air we breathe (Ames, 1979 and 1983; Doll and Peto, 1981; Committee on Diet, Nutrition, and Cancer, 1982; Abelson, 1983; Rogers and Longnecker, 1988). These agents have in common an ability to interact directly with DNA, or to become metabolically activated into substances that can interact with DNA. Presumably, human cancer arises as a consequence of the multiple mutation-like events produced by such interactions, coupled with transient, non-heritable epigenetic events (Cairns, 1981; Weinstein et al., 1984; Weinstein et al., 1985; Klein and Klein, 1985). But if mutation-like events are involved in carcinogenesis, what are the specific targets for the DNA-damaging agents that cause them? In other words, which of the tens of thousands of human genes, if damaged, could move a cell along the path toward malignancy?

The answer began to emerge a decade ago when Weinberg and his co-workers reported the development of an in vitro system for screening DNA from tumor cells for potential cancer-causing genes (Shih et al., 1979). Their system, which involved transfection

of DNA into NIH 3T3 mouse fibroblasts, led to the identification of the first transforming gene, or oncogene. The oncogene they identified was a mutated H-ras gene from the human bladder carcinoma cell line T24 (Duesberg, 1985). Many more dominant-acting transforming genes have been identified during the ensuing ten years (reviewed in Cooper, 1982; Weinberg, 1982; Varmus, 1984; Bishop, 1985 and 1987; Nishimura and Sekiya, 1987). Oncogenes have been found not only in the DNA of spontaneous human tumors, but also in the DNA of tumors induced in animals by chemicals or radiation and in the genomes of acutely-transforming retroviruses.

Molecular analysis reveals that oncogenes are closely related in sequence to normal cellular genes called proto-oncogenes; oncogenes are, in effect, mutant alleles of wild-type proto-oncogenes. Proto-oncogenes have been highly conserved during evolution, consistent with the central role they appear to play in regulating basic processes such as cell division, growth, and differentiation. Such a role also makes proto-oncogenes attractive candidates for targets of environmental carcinogens.

The alteration of a proto-oncogene so that it acquires transforming ability is termed "activation", and oncogenes are simply "activated" proto-oncogenes. (Some researchers prefer to call the normal cellular gene an oncogene and the transforming allele an "activated oncogene".) Proto-oncogenes become activated as a result of changes in DNA that alter their expression or the structure of the protein they produce. At the molecular level, DNA damage may lead to point mutations, large deletions, duplications, or rearrangements in a proto-oncogene that in turn cause increased gene expression, inappropriate or unregulated gene expression, expression of a mutant protein product, or a combination of these effects. For example, ras genes normally become activated by point mutations at a few key sites in their protein coding sequence (Shih and Weeks, 1984; Barbacid, 1987), while myc genes most often become activated by gene amplification, chromosomal rearrangements, or

proviral insertions that increase and/or deregulate myc expression (Kelly and Siebenlist, 1985; Modjtahedi et al., 1985; Kelly, 1986; Yokota et al., 1988; Tsuda et al., 1988).

In the last few years researchers have discovered other genes involved in carcinogenesis whose normal expression prevents or inhibits transformation (Noda et al., 1989). These genes have been dubbed anti-oncogenes or tumor suppressing genes (reviewed in Barbacid, 1987; Friend et al., 1988). Like oncogenes, tumor-suppressing genes are dominant-acting and can be targets for DNA-damaging agents; however, it is damage that inactivates tumor-suppressing genes which contributes to carcinogenesis. As might be inferred, individuals who are heterozygous for a tumor-suppressing gene as a result of inheritance appear to be at increased risk of cancer. For example, one copy of the tumor suppressing gene Rb-1 appears to be inactivated by hereditary deletion in people predisposed to develop familial retinoblastoma (Cavenee et al., 1983; Lee et al., 1987; Friend et al., 1988). If a somatic mutation inactivates the other copy of Rb-1, tumor is likely to develop.

At first glance, the ability of single oncogenes to transform NIH 3T3 fibroblasts appears to contradict the idea of multistep carcinogenesis, but this is an illusion. NIH 3T3 fibroblasts have been passaged extensively in vitro, undergone gross genetic changes (they are tetraploid), and acquired phenotypic characteristics of tumors cells such as an infinite life span (Land et al., 1983b). The idea that a single oncogene can transform normal cells into cancer cells has been rejected by most researchers. Instead, the picture that has emerged is almost identical to that proposed 16 years ago by Comings (1973); namely, that the transformation of normal cells into malignant tumor cells is the result of the activation of multiple oncogenes coupled with the inactivation of one or more tumor suppressing

genes, and that the process is influenced by epigenetic factors as well (Land et al., 1983a; Duesberg, 1985; Klein and Klein, 1985 and 1986; Weinstein et al., 1985; Van Den Hooff, 1986; Nowell, 1988; Friend et al., 1988; Seemayer and Cavanee, 1989).

The ras Gene Family

Of the several dozen different oncogenes identified to date, one family of genes shows up more often than any other: the ras genes (for reviews, see Shih and Weeks, 1984 and Barbacid, 1987). The first oncogene identified a decade ago was an H-ras oncogene from a human bladder carcinoma cell line, and ras oncogenes subsequently have been found in a wide variety of spontaneous and induced tumors of many species. The human ras gene family consists of three genes (H-ras-1, K-ras-2, and N-ras) and two pseudogenes (H-ras-2 and K-ras-1) (Chang et al., 1982b; Hall et al., 1983; McGrath et al., 1983). These genes are located on different chromosomes: H-ras is on chromosome 11, K-ras is on chromosome 12, and N-ras is on chromosome 1. Certain ras genes have been captured by acutely-transforming retroviruses: H-ras and K-ras are the transforming genes of Harvey and Kirsten murine sarcoma viruses, respectively (Ellis et al., 1981; Tsuchida et al., 1982), and this gene family takes its name from these viruses (ras is an acronym for **ra**t **s**arcoma virus).

The three ras genes share a similar structure of 4 exons that code for a 21 kD protein of 189 amino acids ($p21^{ras}$) (Taparowsky et al., 1983). K-ras has two fourth exons and so can code for two distinct p21s (McGrath et al., 1983). K-ras also has a fifth exon (exon 0) located upstream from the 4 exons that code for the p21 (McGrath et al., 1983). A similar upstream exon (exon -1) has recently been found in H-ras (Cichutek and Duesberg, 1986). These exons are transcribed and form part of the processed ras mRNA, but they are not translated; their function is unknown, but they may be involved in

regulating translation of ras mRNA. This suggests that changes within these non-coding exons might alter the level of ras expression.

The protein product of the ras genes has been the focus of several investigations. Translation of ras mRNA gives rise to a precursor polypeptide (pro-$p21^{ras}$) that migrates to the inner surface of the plasma membrane, where it is processed, phosphorylated, and acquires palmitic acid residues (Shih and Weeks, 1984). The specific cellular function of $p21^{ras}$ is unclear, but it has been found to bind GDP and GTP, to have GTPase activity, and to exhibit an autokinase activity (Shih et al., 1982; Shih and Weeks, 1984; Sweet et al., 1984; Lacal and Aaronson, 1986; Lacal et al., 1986). These characteristics, along with the location of the protein on the inner surface of the plasma membrane and its recently reported homology with the alpha subunit of the G-protein transducin (Lochrie et al., 1985), suggest that $p21^{ras}$ functions as a signal transducer, modulating the transfer of messages from receptors on the cell membrane to intracytoplasmic effectors (Gilman, 1984; Kahn and Graf, 1986). In this regard, the observation that ras genes activated by point mutations frequently have reduced GTPase activity suggests one mechanism by which mutation might alter function, leading to perturbed signal transduction (Finkel et al., 1984; Gibbs et al., 1984; Sweet et al., 1984). But the picture is not a simple one since mutations that reduce binding and hydrolysis of GTP do not always increase the transforming ability of ras oncogenes (Lacal et al., 1986) and may actually decrease their transforming ability (Walter et al., 1986).

Theoretical Mechanisms of ras Activation

Observed differences between ras oncogenes and proto-oncogenes have led to two mechanistic theories of ras activation. The quantitative theory holds that increased expression of a normal ras proto-oncogene is sufficient to activate the gene's transforming potential. Such enhanced expression occurs in retroviruses by linkage of the ras gene to a

viral transcriptional enhancer, and might occur in tumor cells by gene amplification, by chromosomal rearrangements that link ras to a more active promoter or bring it in proximity to enhancers, or by mutations within non-coding exons or introns that alter mRNA translation (Bishop, 1987). Support for the quantitative theory comes from experiments in which ras proto-oncogenes linked to viral promoters acquire transforming capability (DeFeo et al., 1981; Chang et al., 1982a; Pulciani et al., 1985; Ricketts and Levinson, 1988), although other studies fail to demonstrate this effect (Tabin and Weinberg, 1985). Cohen and Levinson (1988) recently demonstrated that a mutation within the fourth intron of the T24 H-ras oncogene, which increases expression tenfold, also increases the transforming ability of this gene, although the mutation is not sufficient in itself to activate the H-ras proto-oncogene.

There is some in vivo support for the quantitative theory of ras activation as well. For example, enhanced expression of ras occurs in tumors induced by retroviruses that carry transduced ras oncogenes and in some chemically-induced tumors (Quintanilla et al., 1986). Increased amounts of H-ras mRNA have been detected in several types of human tumors (Tatosyan et al., 1985). Increased expression of H-ras is common in human breast carcinomas (Hand et al., 1984; Lundy et al., 1986; Thor et al., 1986), but alterations in gene sequence are rare (Kraus et al., 1984; Rochlitz et al., 1988). Ohuchi et al. (1987) found increased amounts of H-ras mRNA and p21 in human gastric adenocarcinomas compared to adjacent normal gastric mucosa, but no increased expression of N-ras or K-ras. In a similar study, Noguchi et al (1986) found increased amounts of H-ras p21 in the parietal cells of normal gastric epithelium as well as in most gastric adenocarcinomas. Increased expression of H-ras has been detected in prostatic carcinomas (Rjinders et al., 1985; Viola et al., 1986) and bladder carcinomas (Viola et al., 1985). In both organs, benign proliferative lesions and normal tissue do not have increased expression of H-ras, and a

survey of bladder carcinomas found structural mutations in H-ras in only 2 of 23 tumors (Fujita et al., 1984). Increased amounts of H-ras and K-ras mRNA and ras p21 have been found in colon tumors (Thor et al., 1984; Hand et al., 1984; Spandidos and Kerr, 1984; Gallick, et al., 1985) but ras p21 content was increased only in carcinomas and not in adenomas.

The other theory of ras activation is the qualitative theory, which holds that activation results from changes in proto-oncogene structure that alter the ras protein product. Such changes might be point mutations that cause single amino acid substitutions or frameshifts, or large deletions or rearrangements that cause large changes in the ras protein. Both types of qualitative changes have been found in various viral and cellular oncogenes, although so far only point mutations have been found in ras oncogenes. Structurally altered ras genes have been isolated from a wide range of human tumors (Tabin et al., 1982; Reddy et al., 1982; Santos et al., 1983; Capon et al., 1983a and 1983b; Shimizu et al., 1983; Fujita et al., 1984; Suarez et al., 1987; Neri et al., 1988 and 1989; Bar-Eli et al., 1989). Experimental support for their significance comes from numerous studies with ras oncogenes that acquired transforming capability through point mutations induced in vitro at codons 12 (Fasano et al., 1984; Seeburg et al., 1984; Spandidos and Wilkie, 1984), 13 (Fasano et al., 1984), 59 (Fasano et al., 1984), 61 (Taparowsky et al., 1983; Yuasa et al., 1983; Fasano et al., 1984; Der et al., 1986), 63 (Fasano et al., 1984), or 116 (Walter et al., 1986). Activation also has been produced by small deletions or insertions in the region of codon 12 (Chipperfield et al., 1985).

These two mechanisms of ras activation are not mutually exclusive. In fact, both mechanisms operate in retrovirus-induced transformation (Tabin and Weinberg, 1985), and their additivity has been demonstrated in vitro (Spandidos and Wilkie, 1984; Der et al., 1986; Ricketts and Levinson, 1988). Nor are these two mechanisms necessarily caused by

different agents; either type of activation can be produced by agents that damage DNA. For example, we now know that the T24 H-ras oncogene owes its potent transforming ability not only to a point mutation in codon 12 that alters the structure of the p21, but also to a point mutation in the last intron that increases expression of the altered protein (Cohen and Levinson, 1988).

ras Oncogenes in Animal Tumors

No ras oncogenes have been found in normal cells from cancer patients whose tumor cells contain such genes (Cline, 1989), so the activation of ras genes appears to be the result of somatic mutation. Activated ras genes have been found not only in a wide range of human cancers, but also in spontaneous tumors and tumors induced by chemical carcinogens and radiation in experimental animals (for lists, see Slamon et al., 1984; Vousden and Marshall, 1984; Nishimura and Sekiya, 1987).

Leibovitch et al. (1986) studied expression of all 3 ras genes in 3 related lines of rat myoblasts and found that expression of K-ras positively correlates with malignant behavior. Not only is K-ras mRNA content increased in the most malignant cells, but they contain a new K-ras transcript that is absent from the non-tumorigenic parent cells.

Study of ras oncogenes from induced tumors has revealed some of the molecular mechanisms by which environmental carcinogens may contribute to cancer development. Several studies suggest that activation of ras is involved at an early stage in carcinogenesis. For instance, when Zarbl et al. (1985) gave female rats a single dose of MNU, mammary carcinomas were readily induced, and more than 80% of these tumors carry a specific G-->A transition in codon 12 of H-ras. Guerrero et al. (1984) found a similar G-->A transition leading to activation of K-ras in tumor cells from mice with lymphomas induced by gamma radiation. K-ras activation also accompanies induction of kidney mesenchymal

tumors by methyl(methoxymethyl)nitrosamine (Sukumar et al., 1986) and induction of lung tumors by tetranitromethane (Stowers et al., 1987). Almost all mouse skin tumors initiated with DMBA have a specific A-->T transversion in codon 61 of H-ras, and this change is frequently found even in premalignant papillomas (Balmain and Pragnell, 1983; Bizub et al., 1986; Quintanilla et al., 1986). The frequency of this mutation varies with the initiating agent used rather than the promoter, suggesting that mutation occurs at the time of initiation. There is also an increase in H-ras expression at the mRNA level in papillomas compared to surrounding normal epidermis (Balmain et al., 1984).

Sinha et al. (1988) found N-ras oncogenes in rat liver tumors induced by aflatoxin, as well as in rat hepatocyte cell lines transformed in vitro by aflatoxin treatment. H-ras oncogenes are found in almost 100% of hepatocellular carcinomas in B6C3F_1 mice, whether the tumors arise spontaneously (Reynolds et al., 1986) or are induced by carcinogens (Wiseman et al., 1986; Maronpot et al., 1987; Barbacid, 1987). Moreover, the same H-ras oncogene is found in 40% of adenomas, suggesting that activation is an early event in tumor development. In the case of spontaneous tumors, H-ras is activated by random mutations in codon 61, but in tumors induced by hydroxy-acetylaminofluorine or vinyl chloride the activating lesion in codon 61 corresponds to that normally produced by the respective carcinogen; that is, C-->A transversions in the case of hydroxy-acetylaminofluorine and A-->T transversions in the case of vinyl chloride. On the other hand, when Hsieh et al. (1987) examined liver tumors induced in Sprague-Dawley rats by initiation with DEN and promotion with phenobarbital they found no consistent change in expression of H-ras, although they did not determine if there were structural mutations within H-ras or alterations in the other two ras genes.

ras Oncogenes in Human Tumors

Experimental results implicating ras activation as a frequent and early event in carcinogenesis are supported by at least some studies of spontaneous human tumors. For example, not only do more than one third of human colorectal tumors contain point-mutated K-ras genes, but K-ras oncogenes are found in a high percentage of adenomas as well as carcinomas, suggesting that mutation of ras might occur early in colon carcinogenesis (Bos et al., 1987; Forrester et al., 1987). Almoguera et al. (1988) found point-mutated K-ras genes in a high percentage of pancreatic cancer, too. Spandidos and Kerr (1984) detected increased amounts of H-ras and K-ras mRNA in both benign colon polyps and colon carcinomas, and they concluded that increased ras expression was an early event in colon carcinogenesis. However, Thor et al. (1984) found normal amounts of $p21^{H\text{-}ras}$ in adenomas but increased amounts in carcinomas, and they concluded that increased ras expression was a late event in colon carcinogenesis. H-ras expression (p21 content) is increased in cells of dysplastic lesions of the urinary bladder as well as bladder carcinomas, suggesting that increased ras expression may be an early event in the development of tumors in this location, too (Viola et al., 1985).

On the other hand, Albino et al. (1984) found a mutationally activated N-ras oncogene in only one of five melanoma cell lines derived from separate metastases in a single patient, and they concluded that in this instance the mutation was not involved in the early stages of carcinogenesis. Neri et al. (1988) found that tumor cells from 6/33 patients with acute lymphoblastic leukemia contained mutationally-activated N-ras oncogenes. In five cases, activation is due to a G-->A transition in codon 12 or 13. However, in four patients only a fraction of the tumor cells had the N-ras oncogene, suggesting that N-ras mutation occurred late in tumor development (see Toksoz et al., 1989 for review). Neri et al. (1989) reached the same conclusion regarding N-ras activation in multiple myelomas.

Gerosa et al. (1989) studied ras expression in human glial tumors. They found that N-ras expression is increased in highly malignant glioblastomas and in cell lines derived from them, compared to less malignant gliomas or normal glial cells, implying that enhanced ras expression might be a late event in progression of brain tumors. Fan (1988) found a similar situation in a patient with metastatic prostate cancer, where only 25% of the primary tumor cells had increased $p21^{H\text{-}ras}$ content, but almost all the cells from a vertebral metastasis contained increased p21, suggesting that increased H-ras expression was a late event in this patient's cancer development. Study of $p21^{H\text{-}ras}$ content in tumors from patients with metastatic breast cancer has revealed that ras expression is increased in primary tumors from patients that have metastases compared to primary tumors from those who do not (Lundy et al., 1986), but that ras expression in metastatic tumors is variable and may be higher or lower than that of the primary tumor (Fromowitz et al., 1987). In none of the studies on ras expression was the possibility of point-mutations within ras investigated.

The variability in ras expression or lack of ras mutation in metastatic tumors does not rule out that ras oncogenes are involved in metastasis. Rather, ras oncogenes may assist cells in metastasizing but be unnecessary for tumor growth once a distant site is reached. Egan et al. (1987b) reached exactly this conclusion in experimental studies of metastasis by H-ras-transformed NIH 3T3 fibroblasts. If ras expression is irrelevant to metastatic tumor growth, one might expect it to vary. After all, metastatic tumor cells are a clonal population, and as Heppner (1984) and Schirrmacher (1985) have pointed out, cloned tumor cells are often phenotypically unstable compared to the parent cell population, changing even from one generation to the next.

In Vitro Transformation by ras Oncogenes

Activated ras genes were first identified by their ability to transform NIH 3T3 fibroblasts to focus formation. The initial oncogene so discovered, T24 H-ras, derives its potent transforming ability from no less than three changes: a mutation in codon 12 that alters the structure of the protein (Reddy et al., 1982) coupled with a mutation in the third intron (Cohen and Levinson, 1988) and an upstream enhancer region (Puga et al., 1985), both of which increase expression of the altered p21. Since the discovery of T24 H-ras, investigators have found that NIH 3T3 fibroblasts can be transformed to various phenotypes by ras genes from other tumors (Tainsky et al., 1984) or from oncogenic retroviruses (Cichutek and Duesberg, 1986), ras genes mutated in vitro (Santos et al., 1983; Fasano et al., 1984; Chipperfield et al., 1985; Der et al., 1986; Lacal et al., 1986), and even normal ras proto-oncogenes expressed at a high level (Santos et al., 1983; Cichutek and Duesberg, 1986). The role of ras in human carcinogenesis is difficult to ascertain from these experiments, however. Not only do NIH 3T3 fibroblasts seem exquisitely sensitive to transformation by ras oncogenes, but in most experiments there was also a low frequency of spontaneous transformation in untreated or sham-treated controls. Transfected ras oncogenes and proto-oncogenes are capable of transforming primary rat cells (Land et al., 1983b; Cichutek and Duesberg, 1986) and immortal rat cell lines (Land et al., 1983b; Seeburg et al., 1984; Walter et al., 1986; Ricketts and Levinson, 1988; Cohen and Levinson, 1988), but since rat cells also frequently transform spontaneously in culture (Auersperg et al., 1987), the question remains what part ras genes play in human cell transformation.

Tumorigenic transformation of rodent cells by ras oncogenes is well-documented. Infection of NIH 3T3 fibroblasts (Hamner et al., 1986), rat adrenocortical cells (Auersperg et al., 1981), and mouse bone tissue (Silbermann et al., 1987) with Kirsten murine sarcoma virus makes cells tumorigenic. Roop et al. (1986) and Dotto et al. (1988) found that

infection of mouse keratinocytes with Harvey murine sarcoma virus had a similar effect. Mouse NIH 3T3 fibroblasts and rat cells transformed by transfection with ras oncogenes are almost always tumorigenic (Land et al., 1983b; Spandidos and Wilkie, 1984; Jenkins et al., 1984; Bernstein and Weinberg, 1985; Thorgeirsson et al., 1985; Bolscher et al., 1986; Pozzatti et al., 1986; Wallace et al., 1986; Gao et al., 1987; Storer et al., 1988; Ananthaswamy et al., 1988; Ricketts and Levinson, 1988). So are mouse C3H 10T1/2 cells (Egan et al., 1987a), mouse C127 cells (Muschel et al., 1985), and Chinese hamster lung fibroblasts (Spandidos and Wilkie, 1984; Muschel et al., 1985; Wyllie et al., 1987; Chadwick and Lagarde, 1988). Transfection of T24 H-ras into mouse melanoma cell lines enhances their tumorigenicity (Price et al., 1988). Established rodent cell lines may be especially susceptible to tumorigenic transformation by ras because they have infinite life spans, and the ease with which primary mouse and rat fibroblasts transform to tumorigenicity following ras transfection may reflect the high rate at which these cells spontaneously acquire infinite life spans.

Attempts to transform human cells with ras oncogenes have been successful only rarely. Sager et al. (1983) found no evidence of transformation in human foreskin fibroblasts transfected with the H-ras oncogene from the human bladder carcinoma cell line EJ. However, Sutherland et al. (1985) and Hurlin et al. (1987; Fry et al., 1988) successfully transformed human foreskin fibroblasts to focus formation and anchorage independence by transfection with T24 H-ras, and Wilson et al. (1989a) achieved similar results with the N-ras oncogene from the human leukemia cell line 8402. In each case, the transformed cells have finite life spans and are non-tumorigenic. Pater and Pater (1986) transformed human embryonic kidney cells immortalized by papilloma virus to anchorage independence by transfection with the EJ H-ras oncogene, but apparently did not test the tumorigenicity of the transformed cells.

Evidence from various experimental systems supports the idea that ras oncogenes do play a role in tumorigenic transformation of human cells. For example, Tainsky et al. (1984) attributed enhancement of the tumorigenicity of the human teratocarcinoma cell line PA1 to a spontaneous mutation in N-ras. Agnor et al. (1988) saw similar results following transfection of human breast carcinoma cells with H-ras. There are few reports of tumorigenic transformation of human cells by transfected ras oncogenes. Human bronchial epithelial cells transfected with v-H-ras eventually developed into tumorigenic cells, but only after a long time in culture during which the cells acquired an infinite lifespan (Yoakum et al., 1985). Human fibroblasts (O'Brien et al., 1986) and keratinocytes (Rhim et al., 1985) immortalized by SV40 virus can be transformed into tumor cells by infecting them with Kirsten murine sarcoma virus. Seremetis et al. (1989) malignantly transformed human B-lymphocytes immortalized by EBV with a viral H-ras or tumor-derived N-ras oncogene. Namba et al. (1986, 1988) transformed infinite life span, aneuploid KMST-6 human fibroblasts into tumorigenic cells by infecting them with Harvey murine sarcoma virus or transfecting them with a tumor-derived H-ras oncogene. In our laboratory, diploid MSU-1.1 human fibroblasts, also having an infinite life span, were transformed into tumorigenic cells by transfection with v-K-ras (Fry et al., 1989), T24 H-ras (Hurlin et al., 1989), or a tumor-derived N-ras oncogene (Wilson et al., 1989b).

ras Oncogenes and Tumor Pathology

The relationship between ras genes and pathologic features of tumors in vivo seldom has been explored. This is because tumor histology is not generally used as a measure of carcinogenicity in experimental systems since tumorigenicity itself is taken as a direct indicator of malignancy. However, in clinical tumor pathology, histologic criteria form the chief basis for diagnostic and prognostic decisions. This reliance on histologic criteria rests

on extensive historical evidence for correlations between histologic features and the behavior of human cancer cells. In essence, the microscopic appearance of any tumor -- cytologic features, arrangement of cells, patterns of growth, antigen expression -- describes its biological behavior (Auersperg et al., 1987). Yet little is known about the molecular mechanisms that determine the microscopic features of tumors.

In the case of epithelial cells, Warburton et al. (1986) reported that H-ras-transformed mouse mammary epithelial cells produce "invasive carcinomas", while untransfected cells make only "benign cystadenomas", but the histologic features of the tumors were not further described. Yoakum et al. (1985) transformed human bronchial epithelial cells by transfection with v-H-ras and found that they produce poorly-differentiated malignant tumors whose epithelial nature is revealed only by the prominent intermediate-type intercellular junctions visible with the electron microscope and by positive staining for keratin. Roop et al. (1986) infected mouse keratinocytes with Harvey murine sarcoma virus and found that the cells produce benign papillomas in athymic nude mice. On the other hand, Dotto et al. (1988) infected mouse keratinocytes with Harvey murine sarcoma virus and found that the cells produce carcinomas with various histologic patterns in syngeneic mice. Tumors range from sheets of epithelioid cells to interwoven bands of spindle cells, but all are keratin-positive and vimentin- and Factor VIII-negative. Rhim et al. (1985) infected human keratinocytes with Kirsten murine sarcoma virus and reported that the cells make invasive, rapidly progressive squamous cell carcinomas with characteristic keratin pearls.

In the case of mesenchymal cells, Thorgeirsson et al. (1985) described tumors produced by N-ras- or H-ras-transformed NIH 3T3 fibroblasts as "invasive fibrosarcomas," although Greig et al. (1985) found that untransfected NIH 3T3 fibroblasts also produce invasive spindle cell sarcomas that are histologically indistinguishable from those produced

by ras-transformed cells. The findings of Greig and his colleagues recall earlier experiments by Franks et al. (1971), who examined tumors produced by various mouse cell lines spontaneously transformed in vitro. The cell lines had been established from whole embryos and selected organs from mature mice of two strains. In all, the investigators studied 68 tumorigenic cell lines. They found that the tumors produced by the various cell lines were similar regardless of the organ or tissue from which the cells had been derived, and that all were invasive sarcomas. Franks and his coworkers identified three patterns of growth, which were always mixed together in the tumors: a fibrosarcomatous pattern that was often myxoid, a leiomyomatous pattern that sometimes contained multinuclear giant cells, and a pleomorphic epithelioid pattern that usually contained many multinuclear giant cells. The transition from one pattern to another was gradual instead of abrupt. All the tumors had scant collagen and elastin fibers and, with the exception of the myxoid pattern, mitoses were frequent. Egan et al. (1987a) found that transformation of mouse C3H 10T1/2 cells by T24 H-ras results in morphologically transformed cells whose degree of morphologic transformation in culture is positively correlated with ras mRNA content, but they apparently did not examine the tumors microscopically.

Land et al. (1983b) found that H-ras-transformed primary rat embryo fibroblasts produced small, slow-growing "cartilaginous nodules," while H-ras-transformed infinite life span Rat-1 cells produced "rapidly-growing fibrosarcomas" but their report includes no histologic descriptions or photomicrographs of the tumors. Wyllie et al. (1987) reported that rat fibroblasts transformed by T24 H-ras produce "aggressive fibrosarcomas" that grow rapidly, infiltrate surrounding tissues, produce only sparse collagen, exhibit multifocal necrosis, and have a high mitotic rate. The same cells transformed by the normal H-ras proto-oncogene make similar tumors (although the tumors grow less rapidly), while untransfected cells produce slow-growing "low grade fibrosarcomas" with abundant collagen,

no necrosis, and few mitoses. Still, even these "low grade fibrosarcomas" did invade adjacent adipose tissue, and other investigators have found that untransfected or mock-transfected rat cells often produce invasive tumors (Van Roy et al., 1986; Gao et al., 1987). Storer et al. (1988) found that H-ras-transformed rat embryo cells produce "highly malignant spindle cell tumors, compatible with fibrosarcoma," while the same cells transformed by both H-ras and c-myc produce "malignant neoplasms with little apparent differentiation..., essentially giant cell tumors." Their photomicrograph of a giant cell tumor formed by myc/ras-transformed cells shows a tumor composed of large epithelioid cells with many mononuclear giant cells. The picture is strikingly similar to that of an "undifferentiated fibrosarcoma" produced by H-ras-transformed KMST-6 human fibroblasts (Namba et al., 1986 and 1988). Wyllie et al. (1987) transformed Chinese hamster lung fibroblasts with T24 H-ras and found that the cells produce "aggressive fibrosarcomas" that invaded adjacent fat, skeletal muscle, and dermis.

Auersperg et al. (1981) transformed rat adrenal cortical cells by infection with Kirsten murine sarcoma virus. (Although these cells are derived from glandular tissue, they are mesenchymal in origin.) Of the 18 transformed cell lines these investigators tested for tumorigenicity, 7 produced pleomorphic carcinomas, 6 produced sarcomas, 4 produced mixed tumors (carcinosarcomas) and 1 produced anaplastic tumors. All tumors grow fast and most invade surrounding tissue. In culture, the morphology of the cell lines varies: some lines grow as epithelioid cells and others as spindle-shaped cells, but the type of tumor produced by any cell line is unrelated to its morphology in culture.

In summary, except for the work of Auersperg et al. (1981) and Wyllie et al. (1987), no systematic study has been done to investigate the relationship between ras oncogenes and tumor pathology. Recently, Fry et al. (1989), Hurlin et al. (1989), and Wilson et al. (1989b) succeeded in transforming human fibroblasts with the K-ras, H-ras, and N-ras oncogenes,

respectively. These ras-transformed human fibroblasts are tumorigenic in athymic nude mice, and thus provide the first experimental system for studying the relationship between tumor pathology and specific ras oncogenes in human cells.

ras Oncogenes and Metastasis

The relationship between ras oncogenes and the twin hallmarks of malignancy, invasion and metastasis, has been the subject of numerous investigations in recent years (Mareel and Van Roy, 1986; Liotta, 1988), and was recently reviewed by Nicolson (1987). In a number of experimental systems, ras oncogenes transform cells not just into tumorigenic cells, but into fully malignant tumor cells capable of invading surrounding tissue and metastasizing. However, the degree to which this transformation occurs depends upon the nature of the recipient cell and the level of expression of the ras gene.

Many investigators have utilized mouse NIH 3T3 fibroblasts. After transfection with H-ras, these cells form invasive tumors in athymic nude or histocompatible mice (Thorgeirsson et al., 1985; Bernstein and Weinberg, 1985; Ananthaswamy et al., 1988). Tumors can metastasize spontaneously in athymic nude mice (Thorgeirrson et al., 1985; Bernstein and Weinberg, 1985; Pozzatti et al., 1986; Egan et al., 1987b ; Ananthaswamy et al., 1988) but Wallace et al. (1986) found that they don't always do so, and Bernstein and Weinberg (1985) found that they never metastasize spontaneously in histocompatible mice. When injected intravenously, H-ras-transformed NIH 3T3 fibroblasts form experimental metastases in the lungs of athymic nude or histocompatible mice (Thorgeirsson et al., 1985; Muschel et al., 1985; Bernstein and Weinberg, 1985; Wallace et al., 1986; Bradley et al., 1986; Pozzatti et al, 1986; Egan et al., 1987a and 1987b; Garbisa et al., 1987; Ananthaswamy et al., 1988). Ananthaswamy et al. (1988) found a positive correlation between the level of ras expression in NIH 3T3 fibroblasts and their ability to form spontaneous metastases, but

no correlation between ras expression and experimental metastasis. On the other hand, Egan et al. (1987a) did find a positive correlation between ras expression and experimental metastasis in beige mice and histocompatible mice, and Greenberg et al. (1987) found a similar correlation in athymic nude mice. H-ras-transformed NIH 3T3 fibroblasts invade chicken heart fragments (Bolscher et al., 1986) and human amnion (Thorgeirsson et al., 1985) in vitro and colonize the liver of chick embryos when injected into blood vessels of the chorioallantoic membrane in chicken eggs (Bondy et al., 1985; Hill et al., 1988). Hill et al. (1988) found a positive correlation between the level of ras expression (p21 content) and the ability to colonize the chick embryo.

NIH 3T3 fibroblasts transfected with N-ras (Thorgeirsson et al., 1985) or infected with Kirsten murine sarcoma virus (Hamner et al., 1986; Bolscher et al., 1986) also form spontaneous and experimental metastases in athymic nude mice. Hamner and Starkey (1988) found that metastatic ability of cells infected with Kirsten murine sarcoma virus is negatively correlated with methylation of the v-K-ras oncogene. The ability of ras oncogenes to convey metastatic ability on NIH 3T3 fibroblasts seems to depend on activating mutations, since transfection of the normal proto-oncogene does not make these cells metastatic (Muschel et al., 1985; Garbisa et al., 1987). However, Bradley et al. (1986) reported that H-ras proto-oncogene **could** make NIH 3T3 fibroblasts metastatic, and even untreated or sham-treated NIH 3T3 fibroblasts have proven capable of metastatic tumor formation in some situations (Egan et al., 1987b).

Transfection of ras oncogenes into mouse cell lines other than NIH 3T3 fibroblasts produces results that vary with the recipient cell line. Muschel et al. (1985) found that mouse C127 cells transfected with v-H-ras do not form experimental lung metastases in athymic nude mice, whereas Egan et al. (1987a) found that mouse C3H 10T1/2 cells transfected with T24 H-ras form experimental lung metastases and metastasize

spontaneously, too. In ras-transformed C3H 10T1/2 cells there is a positive correlation between ras mRNA content and the frequency of metastasis.

The relationship between ras oncogenes and metastasis has been studied in several mouse tumor cell lines. Collard et al (1987) transfected non-invasive, non-metastatic BW5147 T-lymphoma cells with T24 H-ras and found that the cells then can invade hepatocyte cultures in vitro and form experimental lung metastases in syngeneic mice in vivo. Invasiveness in vitro correlates positively with ras mRNA content. Warburton et al. (1986) found that mouse mammary epithelial cells, which initially made benign tumors, produced invasive tumors following transfection with T24 H-ras. Kerbel et al. (1987) saw an increase in the frequency of spontaneous metastasis by mouse mammary adenocarcinoma cells transfected with T24 H-ras, and Eccles et al. (1985) saw an increase in the frequency of experimental lung metastasis, too. In contrast, Price et al. (1988) found that mouse melanoma cells transfected with T24 H-ras make more experimental lung metastases in athymic nude or syngeneic mice, but that there is no increase in the rate of spontaneous metastasis. Highly metastatic variants have arisen spontaneously in a mouse lymphoma cell line and a fibrosarcoma line. The metastatic lymphoma variant has an activated K-ras gene which the parental cells lack (Vousden and Marshall, 1984; Vousden et al., 1986), but in the fibrosarcoma cells Alon et al. (1987) found a negative correlation between frequency of metastasis and content of K-ras mRNA and p21.

Rat cells also have been frequent recipients of transfected ras genes. Several investigators found that the T24 H-ras gene converts primary rat cells into cells that spontaneously metastasize and form experimental metastases in athymic nude mice (Muschel et al., 1985; Pozzatti et al., 1986; Garbisa et al., 1987; Alvarez and DeClerck, 1988), and Wyllie et al. (1987) reported similar results with the rat fibroblast line 208F. However, Storer et al. (1988) were unable to produce experimental lung metastases with H-

ras-transfected primary rat cells, although they could do so with cells derived from subcutaneous tumors produced by ras-transfected cells or with cells transfected with both c-myc and H-ras. One possible explanation for these findings is suggested by studies by Kawano et al. (1987) in which they found that the ability of various rat cell lines transformed with the v-fos oncogene to form spontaneous and experimental metastases depends on the manner of integration and level of transcription of the transfected fos gene. Garbisa et al. (1987) and Pozzatti et al. (1986) each found that cotransfection with H-ras and the adenovirus E1a gene inhibited metastasis compared to transfection with H-ras alone. Interpretation of transfection experiments in rat cells is difficult, however, in light of reports by Gao et al. (1987) and Van Roy et al. (1986) that untransfected primary rat cells can invade chicken heart muscle in vitro, make tumors in syngeneic rats and athymic nude mice, and form spontaneous and experimental metastases.

A few rat tumor cell lines have been utilized as recipients of ras genes. Nicolson et al. (1988) transfected T24 H-ras into a non-metastatic mammary adenocarcinoma cell clone, thereby converting the cells into ones that form spontaneous metastases in athymic nude mice. Similar results followed transfection of T24 H-ras into prostatic adenocarcinoma cells by Isaacs et al. (1988).

Transfection of T24 H-ras into Chinese hamster lung fibroblasts converts them into cells that metastasize spontaneously and form experimental lung metastases in athymic nude mice (Spandidos and Wilkie, 1984; Muschel et al., 1985; Wyllie et al., 1987; Chadwick and Lagarde, 1988), although Chadwick and Lagarde (1988) found that untransfected Chinese hamster cells do occasionally form experimental lung metastases as well.

Few studies of ras genes and metastasis have involved non-rodent cells. Sauerbier et al. (1986) investigated the expression of ras genes in a frog renal carcinoma cell line that spontaneously metastasizes at 28°C but not at 18°C. They found that expression of K-

ras (and several other proto-oncogenes) is increased at 28°C compared with 18°C, and thus K-ras expression correlates positively with metastatic behavior in these cells. Only one study has been reported that utilized human cells. Agnor et al. (1988) transfected mutant c-H-ras or v-H-ras into human breast carcinoma cells and found that this had no effect on the frequency of spontaneous metastasis in athymic nude mice.

What might be the molecular mechanisms by which ras oncogenes influence metastasis? Metastasis is not a phenotypic characteristic but a complex process that reflects a spectrum of phenotypes. To metastasize, tumor cells must detach from their neighbors, invade through a vessel wall, survive in the circulation, attach to a vessel wall, traverse a vessel wall again, and proliferate (Lagarde and Kerbel, 1985; Schirrmacher, 1985; Nowell, 1986; Sanchez et al., 1986). There is evidence that ras activation endows cells with several characteristics that are relevant to this process.

Detachment of tumor cells from their fellows appears to reflect changes in cell surface recognition molecules that decrease cell adhesiveness (Buckley, 1985). In particular, an increase in sialic acid residues in fucose-containing glycoproteins on the cell membrane decreases cell adhesiveness and enhances invasion of chick heart fragments in vitro and metastasis in vivo (Roos, 1984; Mareel et al., 1988). Increased sialylation of cell membrane glycoproteins accompanies infection of mouse MO cells with Kirsten murine sarcoma virus and transfection of NIH 3T3 fibroblasts with T24 H-ras (Bolscher et al., 1986).

Once detached, tumor cells must invade tissue and penetrate blood vessel walls. The greatest barrier to their passage is the extracellular matrix, especially basement membranes. Transfection of ras oncogenes into tumor cells decreases fibronectin and collagen production (Warburton et al., 1986; Isaacs et al., 1988), which may be one mechanism by which ras oncogenes enhance invasion and metastasis. Probably more importantly, ras transfection into mouse and rat cells leads to increased production by the cells of a number of proteases,

including collagenases, gelatinase, and Cathepsin L (Denhardt et al., 1987; Alvarez and DeClerck, 1988). In particular, ras oncogenes stimulate cells to produce and secrete more Type IV collagenase which is specifically directed against the type of collagen that is the major component of basement membranes (Turpeenniemi-Hujanen et al., 1984; Thorgeirsson et al., 1985; Garbisa et al., 1987). Liotta et al. (1980) demonstrated a positive correlation between metastatic ability and Type IV collagenase production.

Once in the circulation, cells must survive host defenses, attach at a distant site, escape from a vessel, and proliferate. One of their biggest obstacles is the immune system, especially NK cells and macrophages. Johnson et al. (1985) and Trimble et al. (1986) reported that ras transfection makes cells more susceptible to killing by NK cells in vitro, while Thorgeirsson et al. (1985) and Greenberg et al. (1987) found that ras-transformed cells are not easier for NK cells or macrophages to kill. Studies regarding the effect of ras oncogenes on the ability of cells to colonize and survive in a distant tissue also provide conflicting results. Greenberg et al (1987) found that the ability of mouse C3H 10T1/2 cells injected intravenously to arrest in the lungs reflects their sensitivity to NK cells, and since ras transfection does not make cells less sensitive to NK cells it also does not make cells more likely to colonize the lung. On the other hand, Hill et al. (1988) reported that ras transfection enhances the ability of cells injected intravenously to colonize the liver in chick embryos.

Upon reaching a distant site, cells must proliferate if they are to form a tumor. Greenberg et al. (1987) found that ras transfection enhances the ability of mouse C3H 10T1/2 cells to grow once they colonize the lung, and furthermore, that this ability positively correlates with the level of ras expression. The ability to proliferate may reflect the failure of cells to form gap junctions with adjacent normal cells. Nicolson et al. (1988) recently found that ras transfection of mouse mammary adenocarcinoma cells inhibits gap junction

communication and that this inhibition is accompanied by an increase in the frequency of spontaneous metastasis by the cells.

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THE TUMOR-FORMING CAPACITY OF *RAS*-TRANSFORMED HUMAN FIBROBLASTS

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Running Title: **Tumors by *Ras*-transformed Fibroblasts**

ABSTRACT

Hurlin et al. (14) recently succeeded in malignantly transforming 3 infinite life span human fibroblast lines by transfection with an H-ras oncogene. Malignant transformation of one of these cell lines, MSU-1.1, also has been achieved by transfection with an N-ras (31) or a K-ras (9) oncogene. To find out how closely the experimental system mimicked in vivo carcinogenesis, tumors produced in athymic nude mice by these ras-transformed human fibroblasts were examined microscopically and compared to each other and to spontaneous human soft-tissue sarcomas described in the literature. Comparisons were made of their growth rate, invasive or metastatic behavior, cytologic features, patterns of growth, and immunohistochemical staining reactions. All tumors were progressively-growing, invasive sarcomas, and most were vimentin-positive. Spontaneous metastasis from primary subcutaneous sarcomas was never observed. Sarcomas were classified by morphology as spindle cell sarcomas (with or without whorls), round cell sarcomas, myxoid sarcomas, pleomorphic sarcomas (with mononuclear or multinuclear giant cells), and malignant mesenchymomas. Only K-ras-transformed fibroblasts made myxoid sarcomas. Only H-ras-transformed fibroblasts made spindle cell sarcomas with a whorling pattern or sarcomas that were S-100-positive. Only N-ras-transformed fibroblasts made round cell sarcomas, pleomorphic sarcomas with multinuclear giant cells, or sarcomas that were desmin-positive. These results suggest relationships between specific members of the ras gene family and various histopathologic phenotypic features used by pathologists to classify and assign prognoses to spontaneous human soft-tissue sarcomas. This model system for human cell transformation may prove valuable not only for investigating the molecular

mechanisms involved in carcinogenesis, but also for exploring the molecular basis behind mesenchymal cell differentiation and the histogenesis of soft tissue sarcomas.

Key Words: **H-ras, K-ras, N-ras, transformation, fibroblast, sarcoma**

INTRODUCTION

In order to study human carcinogenesis McCormick and his colleagues have sought to develop an in vitro model system for the malignant transformation of human fibroblasts. Recently, they succeeded in malignantly transforming several infinite life span human fibroblast lines by transfection with ras oncogenes. The infinite life span cell lines were derived from cultures of diploid human fibroblasts that were treated repeatedly with cobalt-60 gamma radiation (KMST-6) (21,22), infected with SV40 virus (GM637), or transfected with a v-myc oncogene (MSU-1.1) (18). These cell lines are non-tumorigenic, but following transfection with a K-ras (9), H-ras (14), or N-ras oncogene (31), clonally-derived strains of ras-transformed fibroblasts formed tumors in athymic nude mice.

To find out how closely this in vitro transformation model mimicked in vivo carcinogenesis, ras-transformed fibroblasts in culture were compared to a spontaneously-transformed human fibroblast line and to cells derived from human sarcomas, some of which also contain ras oncogenes. In vitro, sarcoma-derived, spontaneously-transformed, and ras-transformed fibroblasts were morphologically transformed, formed foci on a cell monolayer, grew in an anchorage independent manner when suspended in soft agar, and had similar growth curves when cultured in medium that lacked exogenous growth factors (9,14,26,31). In the studies reported here, we investigated the tumor-forming capacity of ras-transformed fibroblasts by injecting them subcutaneously into athymic nude mice and examining the tumors microscopically using histochemical and immunohistochemical stains. Specifically, we asked if the particular ras oncogene used to transform the cells influenced the microscopic features or behavior of the tumors they produced. To answer this question, we compared the cytologic features, patterns of growth, and immunohistochemical staining reactions of tumors produced by cell strains derived from a single infinite life span fibroblast

line (MSU-1.1) transformed by 3 different ras oncogenes, each in a different plasmid vector. We also asked if the nature of the infinite life span fibroblast line used as a recipient for the transfected ras oncogene influenced the type of tumor produced. To address this question, we compared tumors produced by cell strains derived from 3 different infinite life span fibroblast lines (MSU-1.1, KMST-6, GM637) transformed by the same H-ras oncogene. Our results indicate that the nature of the recipient fibroblast line and the specific ras oncogene used to tranform the cells did influence the type of tumor produced by ras-transformed human fibroblasts, although other factors such as the plasmid vector, site of integration, and host characteristics appeared to play a role as well.

We expected the tumors to be fibrosarcomas, but many had features reminiscent of other human soft tissue sarcomas, including myxofibrosarcomas, malignant fibrous histiocytomas, neurofibrosarcomas, and myosarcomas. Current uncertainties in the histogenetic classification of human soft tissue sarcomas, and the lack of a specific histochemical or immunologic marker for fibroblastic tumors (discussed in 4,6,7,13,16,19) made it difficult to compare tumors produced by ras-transformed fibroblasts to specific human sarcomas described in the literature. Rather than imply similarities in behavior between our experimental tumors and spontaneous human sarcomas by using names taken from surgical pathology (10), we chose instead to classify the experimental tumors by morphology as spindle cell sarcomas (with and without whorls), round cell sarcomas, myxoid sarcomas, pleomorphic sarcomas (with mononuclear or multinuclear giant cells), and malignant mesenchymomas.

EXPERIMENTAL DESIGN

The ras-transformed cell strains used in these studies were created by Fry et al. (9), Wilson et al. (31), and Hurlin et al. (14) and are listed in Table 1. Tumors were produced by injecting ras-transformed cells subcutaneously into athymic nude mice. Tumor-bearing mice received complete necropsies that included microscopic examination of the tumor itself and major organs and regional lymph nodes for metastases. Samples of tumors were fixed in formalin and B-5, and examined microscopically using various histochemical and immunohistochemical stains. Because immunoperoxidase staining for a variety of antigens has proven useful in classifying soft tissue sarcomas, we screened the tumors produced by ras-transformed fibroblasts using antibodies directed against vimentin and desmin intermediate filaments, S-100 protein, alpha-1-antichymotrypsin, and factor VIII antigen. To investigate the role of the transfected ras oncogene on tumor pathology, tumors produced by various strains of ras-transformed MSU-1.1 fibroblasts were compared. To investigate the role of the recipient cell line on tumor pathology, tumors produced by various H-ras-transformed cell strains were compared.

RESULTS AND DISCUSSION

General Observations

With the exception of a single strain of K-ras-transformed fibroblasts the tumors produced by ras-transformed human fibroblasts were all judged malignant by their progressive rather than self-limited growth, invasive rather than expansive growth, central or multifocal necrosis, and cytologic features such as high cell-to-matrix or nucleus-to-cytoplasm ratio, nuclear atypia, high mitotic index, and multiple large nucleoli. Except as otherwise noted, tumors were not encapsulated and did not compress surrounding tissues. Instead, tumors invaded adjacent adipose tissue and skeletal muscle as they grew. Individual adipocytes or skeletal muscle fibers could be found entrapped within most tumors (see below). Invasion also frequently occurred into the dermis with entrapment of adnexal structures. However, no tumor invaded bone, cartilage, or peripheral nerves, nor did any tumor penetrate into the thoracic cavity. Spontaneous metastases were not found in any tumor-bearing mouse.

That human fibroblasts could be malignantly transformed by transfection of a single activated oncogene does not imply that an activating mutation alone is sufficient for in vitro transformation, or that in vivo carcinogenesis is not a multistep process. On the contrary, in their experiments Fry et al. (9), Hurlin et al. (14), and Wilson et al. (31) introduced ras oncogenes into cells in constructions designed to cause constitutive high expression of the transfected gene. In fact, the transfected oncogene is not highly expressed in the K-ras-transformed cell strains, although it is in the H-ras- and N-ras-transformed cell strains (J. McCormick, personal communication). Thus, at least in the N-ras- and H-ras-transformed cell strains, not only were the ras oncogenes making mutant p21s but they also were producing large amounts of p21 in an unregulated manner in cells that presumably had

two copies of the corresponding normal ras proto-oncogene. In addition, the T24 H-ras oncogene has a point mutation in the third intron (3) that increases its transforming ability. To duplicate this process in vivo would require at least four steps: duplication of a ras gene, an activating point mutation in one copy, a third change leading to increased expression of the mutationally-activated gene, and a fourth change freeing expression from normal regulation. Most likely, whether a transformed cell has one or two normal ras alleles along with a mutationally-activated ras oncogene is unimportant, so duplication is unnecessary. Also, increased and unregulated ras expression may result from a single change, rather than requiring two separate events. Thus, in vitro transformation by ras oncogenes might be duplicated in vivo by as few as two events.

In addition, the cell lines into which ras oncogenes were transfected already had acquired an infinite life span in culture, and evidence from this laboratory (9,14,18,31) and several others (9,14,17,23,24,25,28,31) suggests that this is a requirement for malignant transformation. MSU-1.1 fibroblasts are also partially growth factor-independent, and this also may be important for full malignant transformation.

Tumors produced by ras-transformed fibroblasts were all judged to be sarcomas, and in most cases this was supported by positive staining for vimentin intermediate filaments (Fig. 1). Vimentin intermediate filaments are a general marker for mesenchymal cells, and consequently we expected all tumors to stain positively. Positive staining for vimentin indicated that fixation and processing had been adequate to preserve antigenic determinants of tumor cells. Furthermore, because the antibody we utilized was specific for an epitope on human vimentin filaments and failed to react with murine mesenchymal cells, positive staining for vimentin not only indicated the mesenchymal nature of the tumor cells, but also confirmed that they were of human rather than murine origin. Where possible, only vimentin-positive specimens were examined with the other antibodies. In two cases (cell

lines H-ras-2 and K-ras-1a), alternate specimens were unavailable, so vimentin-negative tissues were utilized.

Transfected Oncogene Influences Tumor Pathology

K-ras-transformed MSU-1.1 Fibroblasts

Cell strains K-ras-1 and K-ras-2 were isolated by Fry et al. (9) as independent foci that appeared in cultures of MSU-1.1 fibroblasts transfected with K-ras. Tumors produced when these cells were injected subcutaneously into athymic nude mice are shown in Table 2. Tumors were classified as myxoid fibromas, myxoid sarcomas, or spindle cell sarcomas.

Cell strain K-ras-1 produced tumors that grew slowly and ceased enlarging when they reached about 1 cm in diameter. Grossly, the tumors were flattened, firm, white nodular masses within the subcutis, but when cut the interior of the nodules was soft. Microscopically, the tumors had a thick fibrous capsule and were composed of small uniform stellate and fusiform cells imbedded in an abundant myxoid matrix containing short wavy collagen fibers. A few small capillaries were scattered throughout the tumor. Cells had a scant amount of eosinophilic fibrillar cytoplasm and oval or reniform nuclei with finely granular chromatin and one or two small nucleoli. Mitoses were scarce (< 1/hpf). Because the tumors did not grow progressively and were encapsulated with no evidence of invasion or cytologic features of malignancy, they were classified as myxoid fibromas.

Cell strain K-ras-2 produced soft, flattened tumors that grew slowly but progressively. Grossly, the tumors were translucent and gelatinous. Microscopically, the tumors resembled the myxoid fibromas just described (Fig. 2), but they were not encapsulated and could be seen invading skeletal muscle. The extracellular matrix was abundant and myxoid. With reticulin stains, numerous thin collagen fibers could be seen surrounding individual cells,

while thicker wavy fibers were scattered throughout the matrix. Mitoses were still scarce (<1/hpf). On the basis of their progressive and invasive growth, these tumors were classified as myxoid sarcomas. Myxoid sarcomas produced by K-ras-2 were negative when stained by the immunoperoxidase method for vimentin, desmin, S-100, alpha-1-antichymotrypsin, or Factor VIII.

Fry et al. (9) assayed each focus-derived K-ras-transformed cell strain for growth in soft agar, and isolated 3 anchorage-independent clones (cell strains K-ras-1a, K-ras-2a, and K-ras-2b). Tumors produced by these cell strains are shown in Table 2. Cell strains K-ras-1a and K-ras-2a, derived from K-ras-1 and K-ras-2 respectively, produced myxoid sarcomas similar to those already described; however, cell strain K-ras-2b produced firm nodular tumors that appeared after a shorter latent period and grew more rapidly. Microscopically, tumors formed by K-ras-2b cells consisted of dense sheets of cells separated into multiple lobules by bands of collagenous stroma, with occasional areas of more loosely-arranged cells within abundant myxoid matrix. In some of these looser areas cells grew in a storiform pattern (Fig. 3). There was extensive central necrosis of individual lobules. Closely-packed cells were round, oblong, or polyhedral with indistinct cell borders and scant cytoplasm. Cells in less dense areas were spindle-shaped and moderately pleomorphic with fibrillar cytoplasm. Nuclei were round or oval with coarsely-clumped chromatin and one or two large nucleoli. Mitoses were uncommon (1-3/hpf). These tumors were classifed as spindle cell sarcomas.

Electron microscopic examination of myxoid sarcomas revealed elongated cells with delicate filamentous cytoplasmic projections arranged in a loose granular matrix that contained bundles of collagen fibers. Cells had numerous polyribosomes; a few profiles of rough endoplasmic reticulum, a few mitochondria, and an occasional Golgi complex could

be visualized in most cells. There was no sign of basement membrane production, and no cell junctions of any kind were seen.

N-ras-transformed MSU-1.1 Fibroblasts

Wilson et al. (31) isolated cell strains N-ras-2, N-ras-3, N-ras-5, and N-ras-8 from four independent foci that arose in cultures of MSU-1.1 fibroblasts transfected with N-ras. Tumors produced by these cell strains following subcutaneous inoculation into nude mice are shown in Table 3. Tumors were classified as spindle cells sarcomas, round cell sarcomas, pleomorphic sarcomas with multinuclear giant cells, or malignant mesenchymomas.

Spindle cell sarcomas were produced by all four N-ras-transformed cell strains and consisted of oblong and spindle-shaped cells arranged in interwoven bundles. Sometimes, short bundles of cells were radially arrayed in a manner reminiscent of the storiform pattern associated with malignant fibrous histiocytomas and dermatofibrosarcoma protruberans. In other tumors, cell bundles intersected at regular angles to form the herringbone pattern commonly associated with human fibrosarcomas (Fig. 4). Such tumors had abundant collagen fibers that were distributed throughout the tumor and appeared to be the product of tumor cells. Still other tumors had less uniform cell bundles that intersected at various angles (Fig. 5). These tumors, which resembled leiomyosarcomas, had a prominent fibrovascular stroma running between bundles of tumor cells, and the hyperchromatic stromal cell nuclei accentuated the fascicular pattern of the tumor. Collagen fibers in these tumors were almost exclusively confined to the stroma, suggesting that they were the product of murine stromal cells rather than the tumor cells. Occasional spindle cell sarcomas contained large, branching vascular channels typical of those described in human liposarcomas and hemangiopericytomas (4).

Cells in spindle cell sarcomas varied from uniform to moderately pleomorphic, but were always closely-packed with scant intercellular matrix. They had a moderate amount of fibrillar eosinophilic cytoplasm with indistinct cytoplasmic margins. Nuclei were elongated, sometimes with blunt ends like those seen in cells of smooth muscle tumors and sometimes with tapered ends more typical of cells in fibroblastic tumors. Chromatin was coarsely-clumped along the nuclear membrane or into strands that spanned the nucleus, and there were one to three nucleoli. The rapid growth of these tumors was reflected in their high mitotic rate (5-10/hpf). Central necrosis was common and also may have been related to the rapid growth rate. Electron microscopic examination of spindle cell sarcomas revealed only oblong cells with numerous polyribosomes, a few profiles of endoplasmic reticulum, and a few mitochondria. Neither intercellular junctions nor bundles of cytoplasmic filaments were observed.

Round cell sarcomas were produced by cell strain N-ras-2 and consisted of dense sheets of fairly uniform cells with no hint of a fascicular pattern (Figs. 6 and 7). Short thin collagen fibers could be seen between tumor cells on silver-stained sections, but no thick collagen fibers were found. Cells were round or polyhedral with indistinct cytoplamic borders. Nuclei were round, oval, or indented and clumping of chromatin along the nuclear membrane gave the nuclei a vesicular appearance. There were one to three small nucleoli. Mitotic activity was similar to the spindle cell sarcomas, and round cell sarcomas also often had necrotic centers.

Cell strain N-ras-3 produced tumors with a distinctive appearance, consisting of very pleomorphic cells arranged in interwoven bundles that sometimes assumed a herringbone pattern. A consistent feature of these tumors was inflammation characterized by multifocal infiltration of neutrophils and lymphocytes, sometimes associated with necrosis of individual tumor cells. These tumors contained a mixture of small spindle cells with oblong nuclei,

rounded cells with a moderate amount of cytoplasm and oval or reniform nuclei, and mononuclear and multinuclear giant cells that had abundant granular eosinophilic cytoplasm (Fig. 8). Giant cells were often elongated. They did not resemble the Langerhans or foreign body type giant cells seen in malignant fibrous histiocytoma, or the osteoclastic giant cells seen in osteosarcomas and Ewing's sarcomas. Although they were sometimes strap-like, they did not have the deeply eosinophilic cytoplasm or myofilaments found in giant cells of rhabdomyosarcomas. These tumors had a lower mitotic rate (1-3/hpf) than tumors produced by other N-ras-transformed cell strains, but bizarre multiplanar mitotic figues were common. There tumors were classified as pleomorphic sarcomas with multinuclear giant cells.

In several tumors produced by cell strain N-ras-3, the pleomorphic sarcoma pattern occurred in association with one of the other patterns of growth. In each of these tumors the two morphologic patterns were sharply demarcated rather than blending together (Fig. 9). Tumors that exhibited two distinct microscopic morphologies were classified as malignant mesenchymomas.

The production of mixed tumors by cell strain N-ras-3 raised the possibility that this strain might be a mixture of two stable cell populations, one that produced spindle cell sarcomas and one that produced pleomorphic sarcomas with multinuclear giant cells. Microscopic examination of N-ras-3 cell cultures revealed scattered multinucleated cells. We attempted to obtain sublines of N-ras-3 that produced only one type of tumor by plating cells at cloning density (100 cells per 100 mm culture dish). When culture dishes were examined two weeks later, individual clones could be differentiated by the presence or absence of multinucleated cells. Ten clones of each type were selected for expansion into cell strains. Two cell strains that contained multinucleated giant cells (N-ras-3.1 and N-ras-3.2) and two cell strains that lacked such cells (N-ras-3.3 and N-ras-3.4) were injected

subcutaneously into 5 mice each. N-ras-3.1 produced malignant mesenchymomas in 2 mice and spindle cell sarcomas in 3 mice, while N-ras-3.2 produced a pleomorphic sarcoma with multinuclear giant cells in one mouse and spindle cell sarcomas in 4 mice. N-ras-3.3 and N-ras-3.4 produced only typical spindle cell sarcomas. These results suggested that N-ras-3 was a mixture of two cell strains, one of which contained only monoculcear cells in culture and produced only spindle cell sarcomas in mice, while the other contained multinuclear cells in culture and sometimes produced pleomorphic sarcomas with multinuclear giant cells or mesnchymomas in mice.

Sarcomas produced by N-ras-2 and N-ras-3 cells stained positively for desmin, including a spindle cell sarcoma, a round cell sarcoma, and a pleomorphic sarcoma with multinuclear giant cells (Table 4 and Fig. 10). Generally there was diffuse pale staining of the entire cytoplasm of tumor cells, compared to the stronger cytoplasmic staining of mouse skeletal muscle fibers and vascular smooth muscle cells within the same section.

H-ras-transformed MSU-1.1 Fibroblasts

Hurlin et al. (14) isolated cell strains H-ras-2, H-ras-3, and H-ras-6 from three independent foci that appeared in cultures of MSU-1.1 fibroblasts transfected with H-ras. Tumors produced when these cells were injected subcutaneously into nude mice are shown in Table 5. Tumors were classified as spindle cell sarcomas (with and without whorls) and malignant mesenchymomas.

Cell strains H-ras-3 and H-ras-6 produced spindle cell sarcomas with a herringbone pattern. The tumors differed from the spindle cell sarcomas produced by N-ras- and K-ras-transformed fibroblasts in three ways. First, scattered among the bundles of spindle cells were rounded nests of closely-packed polyhedral and round cells that did not represent individual fascicles cut in cross-section (Fig. 11). These nests were especially distinct on

silver-stained sections where thin collagen fibers were readily seen surrounding tumor cells in fascicular areas, but were absent within nests of cells. Sometimes the cells in these areas were concentrically arranged, giving the appearance of whorls. Second, thick collagen fibers were more common between cells in these tumors than they were in sarcomas produced by N-ras- or K-ras-transformed cells. Third, the mitotic rate of the tumors was low (1-3/hpf) and necrosis was rarely a feature even of very large tumors. Spindle cell sarcomas produced by H-ras-3 cells stained positively for S-100 antigen (Table 6 and Fig. 12). Staining was diffuse, cytoplasmic, and roughly as intense as that of peripheral nerve fibers on the same section. Approximately one third of the tumor cells were S-100-positive.

Cell strain H-ras-2 produced sarcomas with a variety of microscopic appearances but with one consistent feature; namely, the formation of whorls that sometimes had a small blood vessel or necrotic focus in the center (Fig. 13). These whorls resembled those described in human hemangiopericytomas, meningiomas, and Schannomas. Again, silver stains revealed a lack of pericellular thin collagen fibers within these whorled areas. In two tumors there were large branching vascular channels, a feature also associated with hemangiopericytomas. The remainder of the tumors was a combination of loose myxoid areas, interwoven short fascicles of cells in a storiform pattern, and in one case an area of pleomorphic sarcoma with multinuclear giant cells that led us to classify the tumor as a malignant mesenchymoma. These tumors had a low mitotic rate (1-3/hpf).

Conclusions

Investigation of the relationship between specific ras oncogenes and tumor pathology was complicated by the fact that each of the ras oncogenes used to transform MSU-1.1 fibroblasts was introduced in a different plasmid construction. Attempts to construct a series of plasmids that are identical except for the oncogene they carry are currently underway. Nevertheless, the tumors produced did vary in many microscopic features and in several instances a specific type of tumor was associated with a particular transforming gene:

a) Myxoid sarcomas were produced only by K-ras-transformed MSU-1.1 fibroblasts.

b) Spindle cell sarcomas with a whorling pattern were produced only by H-ras-transformed MSU-1.1 fibroblasts.

c) Round cell sarcomas were produced only by a single strain of N-ras-transformed MSU-1.1 fibroblasts (N-ras-2).

d) Pleomorphic sarcomas with multinuclear giant cells, or mesenchymomas with such a component, were produced only by a single strain of N-ras-transformed MSU-1.1 fibroblasts (N-ras-3).

e) Sarcomas containing desmin intermediate filaments were produced only by N-ras-transformed MSU-1.1 fibroblasts.

f) Sarcomas containing S-100 protein were produced only by a single strain of H-ras-transformed MSU-1.1 fibroblasts (H-ras-6).

Although activation of a specific ras oncogene might cause cells to produce, for example, abundant myxoid matrix or desmin intermediate filaments, such phenotypic features are really the end result of alterations in whole patterns of gene expression.

Regulation of these differentiation programs is undoubtedly complex. Evidence to date suggests that ras gene products are components of signal transduction pathways, probably playing a role similar to G-proteins in modulating signal transmission via transmembrane receptors to intracellular secondary messengers (11,15). That the three ras genes have different sequences suggests that they may play analogous roles in different signal transduction pathways, or function only in certain cell types or at certain periods of development. In such a situation it is reasonable to expect that alterations in ras structure or level of expression would have pleiotropic effects, effects that vary depending upon which ras genes is altered and the nature of the alteration. These studies suggest that changes in K-ras expression may be involved in differentiation of mesenchymal cells toward a phenotype of myxoid matrix production, that altered expression of H-ras may be involved in the program of differentiation that leads cells to arrange themselves in whorls or express S-100 protein, and that altered expression of N-ras may be involved in differentiation programs leading cells to produce desmin filaments, cease collagen production and grow as spheres, or dissociate their nuclear and cytoplasmic division so that multinucleated cells are created.

Our conclusion that the transfected ras oncogene does influence the type of tumor produced by ras-transformed human fibroblasts contrasts with that of Thorgeirsson et al. (30) and Greig et al. (12) who studied mouse NIH 3T3 fibroblasts transformed spontaneously or by transfection with an H-ras or N-ras oncogene. They found that transformed mouse fibroblasts produced poorly-differentiated sarcomas without distinguishing features regardless of the means by which they were transformed. Differences in experimental design make it impossible to ascribe the discrepancy between their results and those we report here solely to the difference between human and mouse cells.

Recipient Cell Influences Tumor Pathology

H-ras-transformed KMST-6 Fibroblasts

KMST-6 fibroblasts transfected with H-ras produced rapidly growing tumors following subcutaneous injection in nude mice (Table 5). Tumors typically had extensive central necrosis accompanied by suppurative inflammation, and mice bearing such tumors had splenomegally due to extramedullary myelopoiesis. Tumors were composed of very pleomorphic cells arranged in solid sheets and separated into small clusters by a scant stroma, an arrangement made prominent by infiltrating neutrophils that accumulated in the stroma and by congestion of stromal capillaries (Fig. 14). Collagen fibers were scarce and confined to the stroma; there was no evidence of collagen production by tumor cells. Cells were round or polyhedral with abundant eosinophilic cytoplasm and distinct cell borders. Nuclei were of various shapes and frequently indented, with coarsly-clumped chromatin and multiple large nucleoli. Mononuclear giant cells with deeply lobulated nuclei were conspicuous. Mitoses were common (5-10/hpf) and bizarre mitotic figures were found in every tumor. These tumors were classified as pleomorphic sarcomas with mononuclear giant cells.

Sarcomas produced by H-ras-transformed KMST-6 fibroblasts contained scattered cells that stained positively for alpha-1-antichymotrypsin (Table 6), but this observation was difficult to interpret. As already mentioned, these tumors were usually necrotic and secondarily inflamed, and it was impossible to ascertain whether the alpha-1-antichymotrypsin-positive cells were tumor cells or host macrophages that had infiltrated the tumor.

H-ras-transformed GM637 Fibroblasts

GM637 fibroblasts transfected with H-ras produced tumors that were similar in microscopic appearance to those produced by ras transformed KMST-6 fibroblasts. Again, tumors developed rapidly following subcutaneous injection in nude mice. As with the KMST-6 tumors, necrosis and suppurative inflammation were prominent features. Unlike the KMST-6 tumors, however, pleomorphic tumor cells more often assumed a spindle shape and were arranged in short interwoven bundles with a prominent collagenous matrix (Fig. 15). These tumors also were classified as pleomorphic sarcomas with mononuclear giant cells. Sarcomas produced by H-ras-transformed GM637 fibroblasts were vimentin-positive but negative for desmin, S-100, alpha-1-antichymotrypsin, and Factor VIII.

Conclusions

The nature of the recipient cell line did affect the type of tumor produced following ras transfection, as illustrated by the differences we observed in tumors produced by MSU-1.1, KMST-6, and GM637 fibroblasts transformed by the identical H-ras oncogene. All three H-ras-transformed cell strains express similar amounts of $p21^{H\text{-}ras}$ (14). Previous studies have shown that the nature of the recipient cell line also influences the tumor-forming capacity of ras-transformed mouse cells (20). However, Franks et al. (8) found that a series of spontaneously-transformed mouse fibroblast lines derived from different organs produced tumors with similar microscopic features suggesting that, in mice, the origin of a cell line does not always influence the type of tumor produced.

Other Factors Influence Tumor Pathology

The transfected oncogene and recipient cell line were not the only factors that influenced what type of tumor a ras-transformed human cell strain produced. Differences in the tumors produced by various lines of N-ras-transformed MSU-1.1 fibroblasts suggested

that factors such as the site of integration of the transfected ras oncogene also might influence tumor type. Integration site could directly influence the expression of the oncogene itself, of adjacent normal cellular genes, or both. Our results are similar to those of Auersperg et al. (1) who found that transformed cell strains derived from a single line of rat adrenal cortical cells infected with Kirsten murine sarcoma virus produced several different types of tumors. Of 18 transformed cell strains they isolated in vitro and tested for tumorigenicity, 7 produced pleomorphic carcinomas, 6 produced sarcomas, 4 produced mixed tumors (carcinosarcomas), and 1 produced anaplastic tumors.

Host factors, too, appeared to influence the type of tumor produced, as was the case with cell strain N-ras-2 that produced both round cell sarcomas and spindle cell sarcomas, or cell strain N-ras-3 that produced giant cell sarcomas, spindle cell sarcomas, or mixed tumors. We were able to establish cell strains derived from N-ras-3 that produced only spindle cell sarcomas, but not strains that produced only pleomorphic sarcomas. One possible explanation for our observations is that variants capable of producing a pleomorphic sarcoma appear within the N-ras-3 strain at some low frequency that is influenced by external factors. In vivo, such factors could include the age, sex, nutritional status, health status, and hormonal status of the host mouse. Another external factor that could influence the type of tumor produced is the microenvironment to which the cells are exposed. For example, the microscopic appearance of tumors produced by a particular ras-transformed cell strain was found to differ when the cells were injected intraperitoneally instead of subcutaneously (5).

Activated or inappropriately expressed ras oncogenes have been found in a wide variety of human tumors, including sarcomas (2,27). Taken together with the results of in vitro studies on transformation of rodent cells by ras oncogenes, the prevalence of ras oncogenes in human tumors strongly implicates ras oncogenes in human carcinogenesis.

The role individual ras oncogenes play in influencing pathologic features of human tumors seldom has been studied, chiefly because a suitable experimental approach has been unavailable. But with the development of ras-transformed human cell strains by Fry et al. (9), Hurlin et al. (14), and Wilson et al. (31) it is now possible not only to study directly the role of ras oncogenes in malignant transformation of human cells, but also to investigate the relationships between specific ras oncogenes and various histopathologic features used by pathologists to classify and assign prognoses to spontaneous human soft tissue sarcomas. This experimental model of human carcinogenesis developed by McCormick and his colleagues will likely prove valuable for exploring the molecular basis behind mesenchymal cell differentiation and the histogenesis of soft tissue sarcomas.

METHODS

Cell Lines

Creation of ras-transformed cell strains by transfection of infinite life span human fibroblast line MSU-1.1 with plasmids containing a K-ras, H-ras, or N-ras oncogene has been described elsewhere (9,14,31). MSU-1.1 fiboblasts were created in McCormick's laboratory (18); they are a non-tumorigenic cell line derived from human foreskin fibroblasts transfected with a v-myc oncogene. MSU-1.1 fibroblasts carry and express both the v-myc gene and a neomycin resistance gene. Transfection of MSU-1.1 fibroblasts with a viral K-ras oncogene, the H-ras oncogene from the human bladder carcinoma cell line T24, or the N-ras oncogene from the human leukemia cell line 8402 (29) resulted in morphologically-transformed cells that could be identified as foci on a cell monolayer or as anchorage independent colonies in soft agar. Several clones of morphologically-transformed MSU-1.1 fibroblasts that contain and express each of the three ras oncogenes were isolated, and these cell strains were utilized in the studies reported here.

Hurlin et al. (14) also transformed two other infinite life span fibroblast lines by transfection with T24 H-ras. KMST-6 fibroblasts were obtained from Namba; they are derived from human embryo fibroblasts treated with ^{60}Co gamma radiation (21,22). GM637 fibroblasts were obtained from the Camden, NJ; they are derived from adult dermal fibroblasts infected with SV40 virus. Neither cell line is tumorigenic. Stable transformants were obtained by transfection of KMST-6 or GM637 fibroblasts with a plasmid containing the H-ras oncogene linked to a neomycin resistance gene, followed by growth in selective medium containing G418 (14)

Assay for Tumorigenicity in Nude Mice

BALB/c athymic nude mice from a closed colony were utilized as tumor hosts in all experiments. Serum from heterozygous female breeders was tested regularly by Microbiological Associates (Rockville, MD) and was consistently free of antibodies to K virus, polyoma virus, murine hepatitis virus, ectromelia virus, GD VIII virus, murine adenovirus, lymphocytic choriomeningitis virus, minute virus of mice, Sendai virus, pneumonia virus of mice, reovirus type 3, EDIM virus, murine cytomegalovirus, Car bacillus, and Mycoplasma pulmonis. Mice were maintained in sterile isolators illuminated by fluorescent light, with a 12 hour/12 hour light/dark cycle, temperature of 70-75°F, and humidity of 30-50%. They were housed in plastic cages at up to five mice per cage. Cages had pine shaving bedding and individual water bottles. Mice were fed pelleted laboratory chow.

Mice of both sexes were used as tumor hosts. Prior to injection, mice were X-irradiated at a dose of 150-300 rads/mouse. From 1 x 10^6 to 5 x 10^6 transformed cells were injected into the subcutis on the dorsal midline of 6-10 week old mice. Afterwards, mice were examined weekly for tumor development. Mice were killed by CO_2 inhalation when tumors were 1-3 cm in diameter, or sooner if the tumor became ulcerated or the mouse showed signs of illness such as listlessness or weight loss.

Collection and Processing of Tissues

Tumor-bearing mice received a complete necropsy that included gross and microscopic examination of at least the tumor itself, axillary, cervical, and mesenteric lymph nodes, thyroid gland, parathyroid glands, adrenal glands, salivary glands, trachea, lungs, heart, liver, spleen, pancreas, and kidneys. In some mice, the digestive tract, reproductive tract, or brain were also examined. Specimens for routine microscopic examination were

fixed in neutral buffered 10% formalin. Sections of tumor for immunoperoxidase staining were fixed in freshly-prepared B-5 fixative for 2 hours, then transfered to 70% ethanol. Small pieces of tumor (1 mm^3) for electron microscopic examination were fixed in cold 4% glutaraldehyde for 2 hours, then transfered to sucrose/phosphate buffer. Sections of fresh tumor were also collected in Eagle's minimal essential medium. Fresh tumor tissue was minced and returned to culture. Since all ras-transformed cell strains carry the neo gene coding for G418 resistance, cells were refed with selctive medium containing G418 after 48 hours to kill any contaminating mouse cells.

Tissues fixed in formalin and B-5 were processed by routine histologic methods and imbedded in paraffin blocks. Tumors were serially sectioned into 2-3 mm slices and all slices were imbedded, so that 3-10 sections of each tumor, representing different areas, wereexamined microscopically. Six micron sections were cut and stained with hematoxylin and eosin for general examination. Additional sections of formalin-fixed tissue were cut and stained with Gomori's trichrome stain or silver stain to demonstrate thick and thin collagen fibers, or Alcian blue or Toluidine Blue to demonstrate mucopolysaccharides. In some cases frozen sections of formalin-fixed tissue were prepared and stained for lipid with Oil Red O.

Additional sections of B-5-fixed tissues were stained by an avidin-biotin immunoperoxidase method for vimentin, desmin, S-100, alpha-1-antichymotrypsin, and Factor VIII antigen. Slides were stained in batches of 18, each batch including a known positive human tissue control and a negative primary antibody control. Briefly, 3 micron sections were placed on poly-L-lysine coated slides and deparaffinized overnight at 60°C. Slides were de-Zenkerized, rinsed, and immersed in methanol for 10 minutes. Endogenous peroxidase activity was blocked by incubation for 10 minutes in H_2O_2, followed by 5 minutes in normal goat serum. Slides were then labelled with one of five primary antibodies: monoclonal mouse anti-vimentin, monoclonal mouse anti-desmin, polyclonal rabbit anti-S-

100, polyclonal rabbit anti-alpha-1-antichymotrypsin, and polyclonal rabbit anti-Factor VIII (all from Lipshaw, Detroit, MI). After treatment with the appropriate linking antibody and labelling reagant, slides were exposed to chromogenic substrate for 5-20 minutes. Slides were counterstained with Mayer's hematoxylin.

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Table 1. Origin of ras-transformed Human Cell Strains

Recipient Cell Line	Transforming Oncogene	Cell Strains Derived	Reference
MSU-1.1	K-ras	K-ras-1, K-ras-1a, K-ras-2, K-ras-2a, K-ras-2b	9
	N-ras	N-ras-2, N-ras-3, N-ras-5, N-ras-8	31
	H-ras	H-ras-2, H-ras-3, H-ras-6	14
KMST-6	H-ras	K1-T1	14
GM637	H-ras	SV-T1	14

Table 2. Tumors Produced by K-ras-transformed MSU-1.1 Human Fibroblasts

Cell Strain	Tumors Examined	Mean Latent Period	Tumor Classification
K-ras-1	2	70 days	Myxoid fibroma
K-ras-2	2	35 days	Myxoid sarcoma
K-ras-1a	8	37 days	Myxoid sarcoma
K-ras-2a	2	39 days	Myxoid sarcoma
K-ras-2b	7	34 days	Spindle cell sarcoma

Table 3. Tumors Produced by N-ras-transformed MSU-1.1 Human Fibroblasts

Cell Strain	Tumors Examined	Mean Latent Period	Tumor Classification
N-ras-2	4	34 days	Round cell sarcoma (1/4) Spindle cell sarcoma (3/4)
N-ras-3	10	19 days	Pleomorphic sarcoma with multinuclear giant cells (5/10) Malignant mesenchymoma (4/10) Spindle cell sarcoma (1/10)
N-ras-5	6	36 days	Spindle cell sarcoma
N-ras-8	8	29 days	Spindle cell sarcoma

Table 4. Immunoperoxidase Staining of Tumors Produced by N-ras-transformed MSU-1.1 Human Fibroblasts[a]

Cell Strain	Type of Tumor	Vimentin	Desmin	S-100	Alpha-1-anti chymotrypsin	Factor VIII
N-ras-2	Spindle cell sarcoma	+	+	-	-	-
N-ras-2	Round cell sarcoma	+	+	-	-	-
N-ras-3	Pleomorphic sarcoma	+	+[b]	-	-	-
N-ras-5	Spindle cell sarcoma	+	-	-	-	-
N-ras-8	Spindle cell sarcoma	+	-	-	-	-

[a]Two tumors of each type were examined except for the round cell sarcoma produced by cell strain N-ras-2, where only a single tumor was examined, and pleomorphic sarcomas produced by N-ras-3, where four tumors were examined. For each tumor, 2-4 sections were stained and examined.

[b]Three of four tumors examined had desmin-positive cells, but none could be found in the fourth tumor.

Table 5. Tumors Produced by H-ras-transformed Human Fibroblasts

Cell Strain	Tumors Examined	Mean Latent Period	Tumor Classification
H-ras-2	10	30 days	Spindle cell sarcoma with whorls
H-ras-3	8	30 days	Spindle cell sarcoma
H-ras-6	5	34 days	Spindle cell sarcoma (4/5) Malignant mesenchymoma (1/5)
K1-T1	5	25 days	Pleomorphic sarcoma with mononuclear giant cells
SV-T1	5	106 days	Pleomorphic sarcoma with mononuclear giant cells

Table 6. Immunoperoxidase Staining of Tumors Produced by H-ras-transformed Human Fibroblasts.

Cell Strain	Type of Tumor	Vimentin	Desmin	S-100	Alpha-1-anti chymotrypsin	Factor VIII
H-ras-2	Whorled sarcoma	-	-	-		
H-ras-3	Spindle cell sarcoma	+	-	-	-	-
H-ras-6	Spindle cell sarcoma	+	-	+	-	-
K1-T1	Pleomorphic sarcoma	+	-	-	+[a]	-
SV-TI	Pleomorphic sarcoma	+	-	-	-	-

[a] Scattered positive cells within the tumor may have been mouse macrophages.

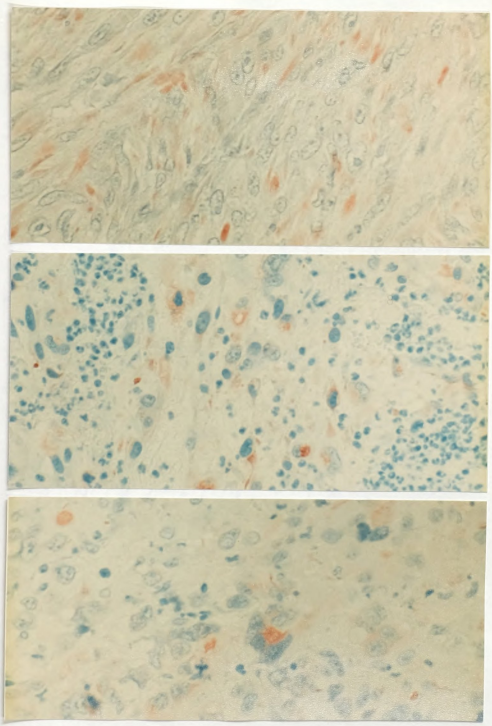

Figure 1. Sarcomas stained for vimentin intermediate filaments. a) N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-5). b) H-ras-transformed KMST-6 fibroblasts. c) H-ras-transformed GM637 fibroblasts (all 500x).

Figure 2. Myxoid sarcoma produced by K-ras-transformed MSU-1.1 fibroblasts (cell strain K-ras-1a). Note abundant extracellular matrix rich in collagen fibers (HE, 500x).

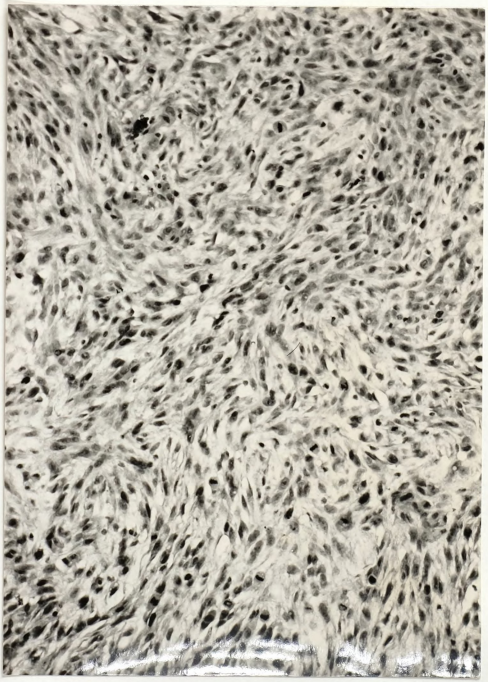

Figure 3. Spindle cell sarcoma produced by K-ras-transformed MSU-1.1 fibroblasts (cell strain K-ras-2b). Cells are arranged in short interwoven fascicles that create a storiform pattern. (HE, 200x).

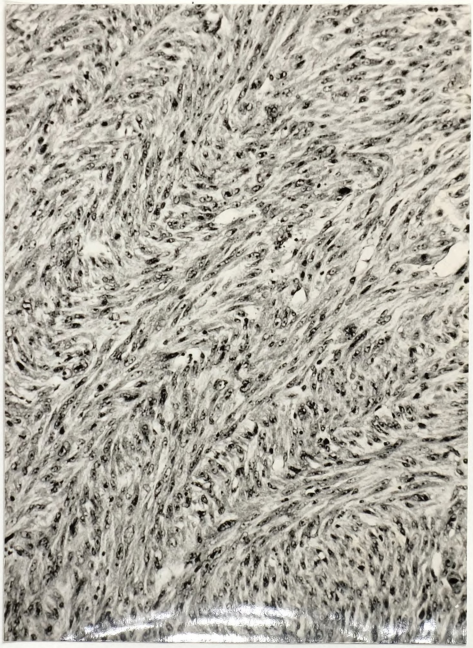

Figure 4. Spindle cell sarcoma produced by N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-8). Note moderate cellular pleomorphism and prominent herringbone pattern (HE, 200x).

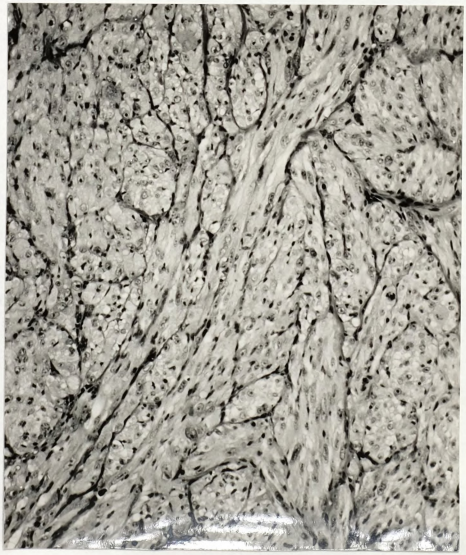

Figure 5. Spindle cell sarcoma produced by N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-3). The fascicular pattern is accentuated by stromal blood vessels that surround and delineate individual tumor cell bundles (HE, 200x).

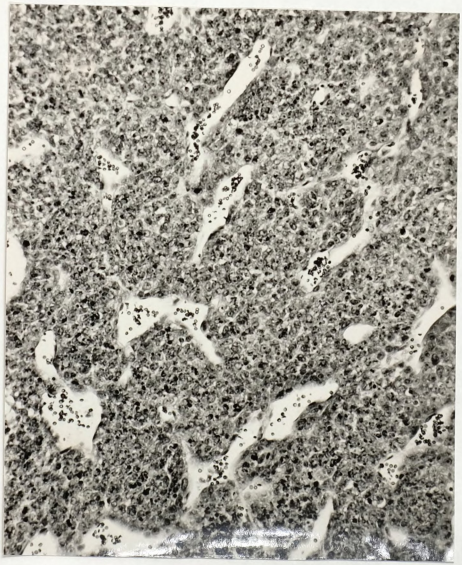

Figure 6. Round cell sarcoma produced by N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-2). Note absence of a fascicular pattern and numerous large vascular channels (HE, 200x).

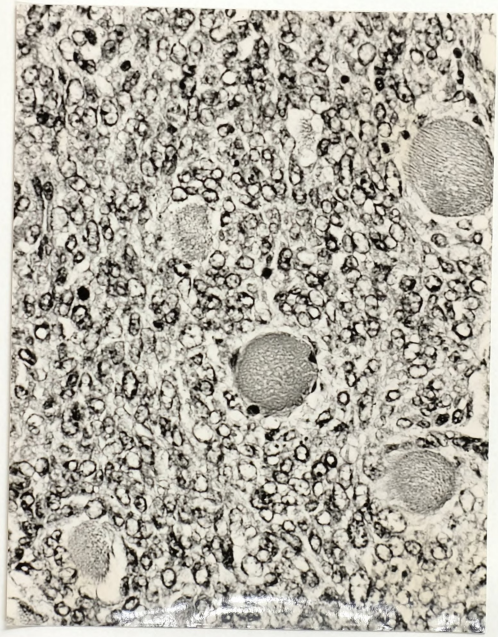

Figure 7. Round cell sarcoma produced by N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-2). Note cross-sections of several skeletal muscle fibers (HE, 500x).

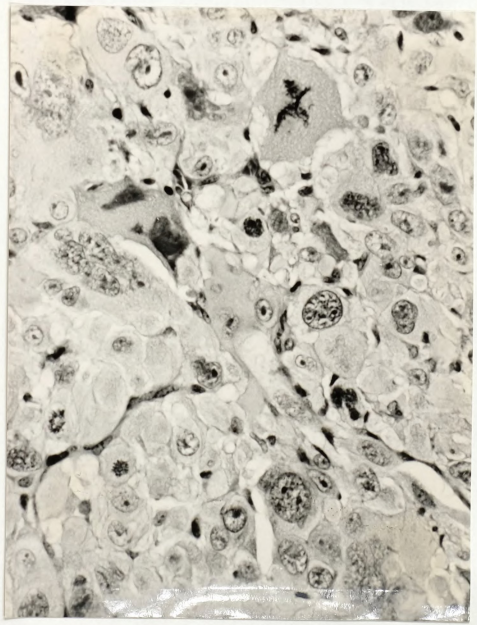

Figure 8. Pleomorphic sarcoma with multinuclear giant cells produced by N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-3). Note abnormal mitotic figure (HE, 500x).

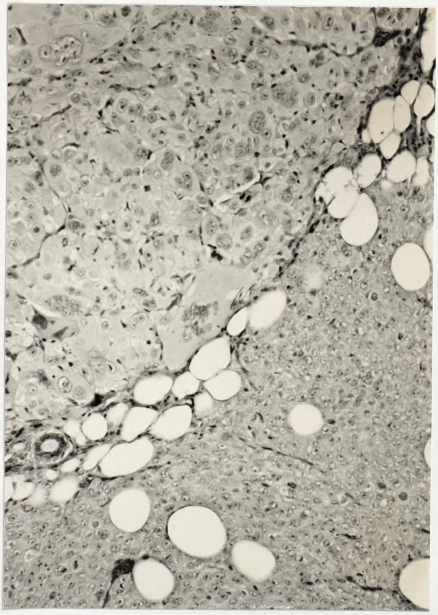

Figure 9. Malignant mesenchymoma produced by N-ras-transformed MSU-1.1 fibroblasts (cell strain N-ras-3). There is a sharp demarcation between the monomorphic round cell pattern and pleomorphic multinucleated giant cell pattern. Note entrapped adipocytes and mammary gland duct (arrow) (HE, 200x).

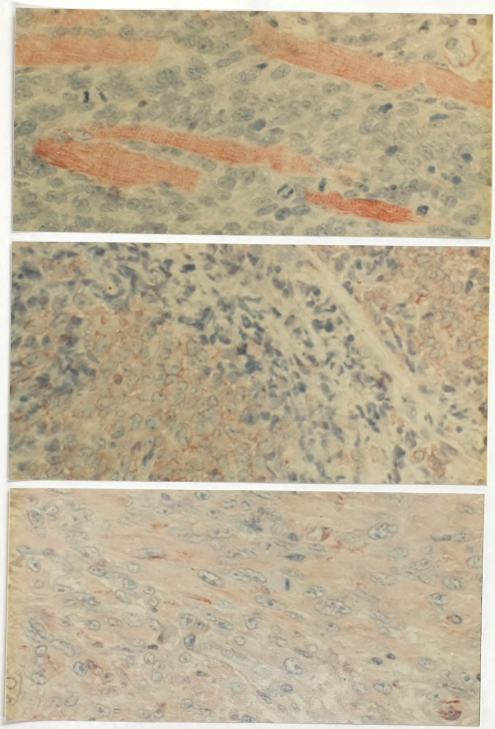

Figure 10. Sarcomas produced by N-ras-transformed-fibroblasts stained for desmin intermediate filaments. a) Cells of tumor formed by cell strain N-ras-5 are desmin-negative, but note staining of mouse skeletal muscle fibers and arteriolar smooth muscle. b) Desmin-positive round cell sarcoma (cell strain N-ras-2). c) Desmin-positive spindle cell sarcoma (cell strain N-ras-2) (all 500x).

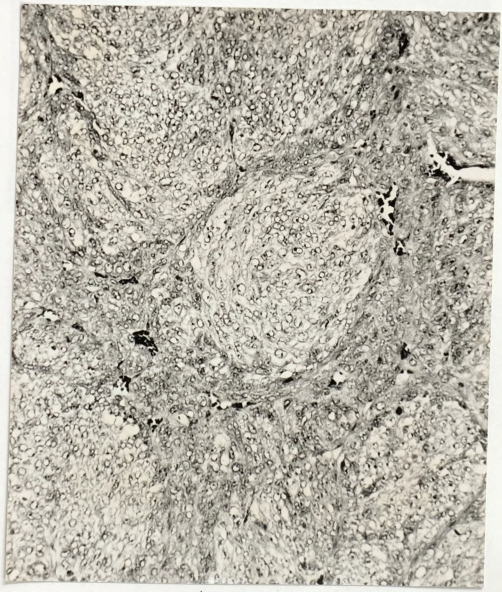

Figure 11. Spindle cell sarcoma produced by H-ras-transformed MSU-1.1 fibroblasts (cell strain H-ras-3). Note rounded clusters of cells with pale cytoplasm that appear to be arranged concentrically (HE, 200x).

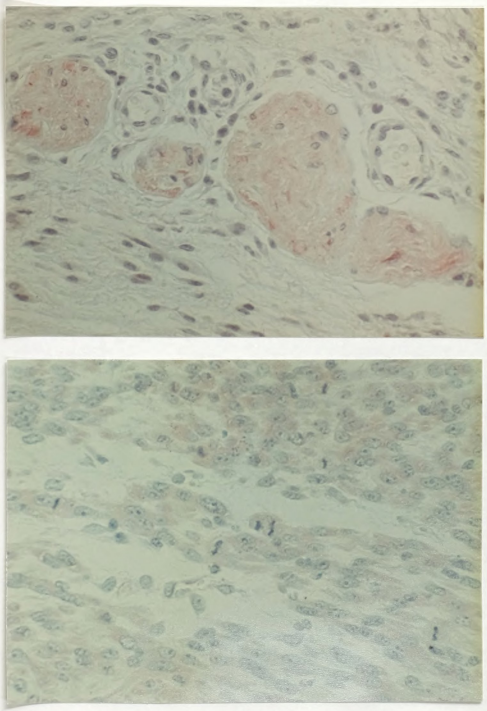

Figure 12. Sarcoma produced by H-ras-transformed fibroblasts stained for S-100. a) Mouse peripheral nerve at edge of tumor; compare intensity of S-100-positive reaction to diffuse cytoplasmic staining of tumor cells in b) Spindle cell sarcoma formed by cell strain H-ras-6 (both 500x).

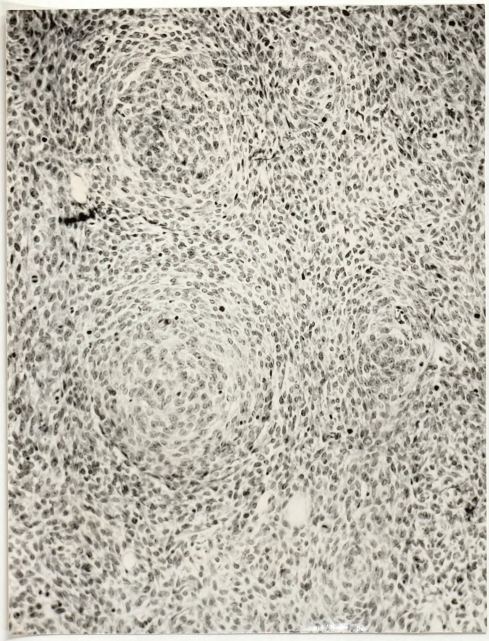

Figure 13. Spindle cell sarcoma produced by H-ras-transformed MSU-1.1 fibroblasts (cell strain H-ras-2). Note fairly uniform cells arranged in multiple whorls (HE, 200x).

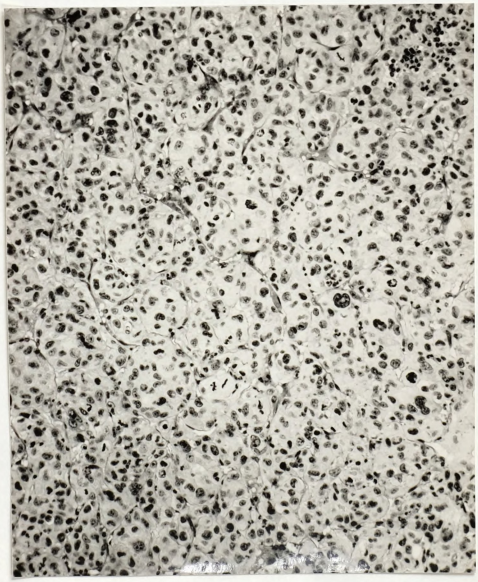

Figure 14. Pleomorphic sarcoma with mononuclear giant cells produced by H-ras-transformed KMST-6 fibroblasts. Cells are separated into small clusters by a delicate fibrovascular stroma. Note giant cell (HE, 200x).

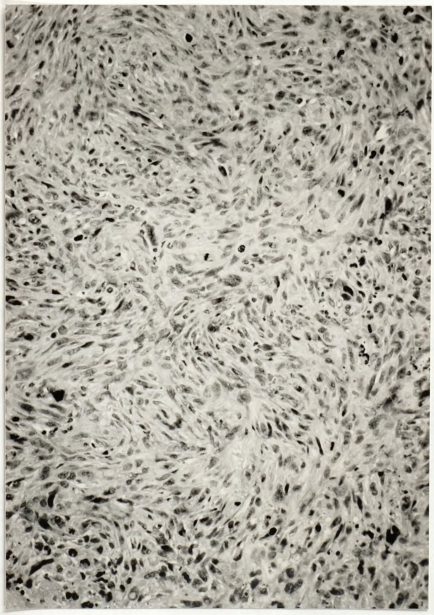

Figure 15. Pleomorphic sarcoma with mononuclear giant cells produced by H-ras-transformed GM637 fibroblasts. Note pleomorphic spindle cells arranged in a storiform pattern (HE, 200x).

TUMORS FORMED BY HUMAN SARCOMA-DERIVED CELL LINES AND A SPONTANEOUSLY-TRANSFORMED FIBROBLAST LINE

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ABSTRACT

Several transformed human cell strains that produce sarcomas in athymic nude mice have been developed in vitro by transfection of infinite life span human fibroblasts with ras oncogenes. To clarify how closely this in vitro model mimics in vivo carcinogenesis, we examined tumors produced in mice by six human sarcoma-derived cell lines, by a clonally-derived subline of one of them, and by a spontaneously-transformed human fibroblast line. One of the sarcoma-derived cell lines (SW-982) proved non-tumorigenic. Tumors produced by the other cell lines were examined microscopically and characterized by their cytologic features, patterns of growth, and immunohistochemical staining reactions. Tumors grew progressively and were invasive, but none metastasized from the subcutaneous injection site. All were classified as sarcomas by their microscopic appearance and positive staining for vimentin, but they differed. The spontaneously-transformed cell line (VIP:F-T) produced spindle cell sarcomas with a herringbone pattern and abundant collagen. Three sarcoma-derived cell lines (8387, SHAC, and NCI) and a subline of one of them (HuT-14) produced spindle cell sarcomas composed of short bundles of cells that intersected randomly, and tumors from all but one of these cell lines (SHAC) had abundant collagen. A fourth sarcoma-derived cell line (HT1080) produced round cell sarcomas without collagen, and a fifth (SW-684) produced a pleomorphic sarcoma with mononuclear giant cells and multifocal necrosis. Tumors produced by these transformed cell lines resembled spindle cell and round cell sarcomas produced by ras-transformed human fibroblasts in rate of growth, cytologic features, morphologic patterns of growth, and failure to spontaneously metastasize from the subcutis. In addition, one sarcoma-derived cell line (SHAC) produced tumors that expressed desmin and a subline of another sarcoma-derived cell line (HuT-14) produced tumors that were S-100-positive, just as several strains of ras-transformed human fibroblasts

do. These results demonstrate the phenotypic diversity of cell lines reportedly derived from tumors of the same histopathologic type, and support the relevance of in vitro transformation of human fibroblasts by transfected ras oncogenes as a model for studying human carcinogenesis.

INTRODUCTION

Fry et al. (*1*), Hurlin et al. (*2*), and Wilson et al. (*3*) recently succeeded in transforming infinite life span human fibroblasts to tumorigenicity by transfection of ras oncogenes. The sarcomas produced in athymic nude mice by these cells ras-transformed cell strains were characterized by cytologic features, patterns of growth, and immunohistochemical staining pattern into spindle cell sarcomas (with and without whorls), myxoid sarcomas, round cell sarcomas, pleomorphic sarcomas (with mononuclear or multinuclear giant cells), and malignant mesenchymomas (*4*). To clarify how closely in vitro transformation of human fibroblasts by ras oncogenes mimics in vivo carcinogenesis, we examined tumors produced by six cell lines reportedly derived from human fibrosarcomas, a clonally-derived subline of one of them, and a cell line spontaneously transformed in vitro and compared the results with what we and our colleagues found previously with tumors produced by the various ras-transformed cell strains. Two of the cell lines (HT1080 and SHAC) were known to contain activated N-ras oncogenes (*5,6*).

The human sarcoma-derived cell lines and spontaneously-transformed cell line were assessed for tumorigenicity by subcutaneous injection into athymic nude mice. Tumors produced by these cells were examined microscopically using chemical and immunohistochemical stains and characterized by the same criteria applied to classify the tumors produced by ras-transformed fibroblasts. The objective was to determine how tumors produced by sarcoma-derived or spontaneously-transformed human fibroblasts resembled those produced by ras-transformed human fibroblasts.

MATERIALS AND METHODS

Cell Lines

The sarcoma-derived cell lines and their derivation are listed in Table 1. The SHAC cell line was derived from a gastric fibrosarcoma that arose in a 58-year old patient (*6*). The HT1080 cell line originally was reported to be derived from a fibrosarcoma that arose near the acetabulum of a 35-year old man who later died of metastatic disease (*7*), but a recent paper states that this line was derived from a metastatic tumor in the mediastinum (*8*). The 8387 cell line reportedly was derived from a fibrosarcoma in the leg of a 25-year old woman (*9,10,11*), but according to Fogh et al., (*12*), the tumor actually may have been an osteosarcoma rather than a fibrosarcoma. The HuT-14 cell line was reported to be derived from a focus of transformed cells arising after treatment of a normal human fibroblast cell line (KD) with 4-nitroquinoline-1-oxide (*13*). The designation HuT-14 was not used in that paper, but was used in subsequent studies of these cells (*14,15*). Molecular analysis has revealed that HuT-14 cells did not arise from the KD cell line (*16*) but instead are derived from the 8387 cell line (*17*). The SW-982 and SW-684 cell lines were derived from fibrosarcomas in separate patients. SW-982 cells were taken from an axillary tumor in a 25-year old woman (*12*; Jorgen Fogh, personal communication), while the SW-684 cells were obtained from a recurrent tumor at an unspecified site in a 68-year old man (*18*; Jorgen Fogh, personal communication). The NCI cell line reportedly was derived from a fibrosarcoma, but details are unknown (C. Cooper, personal communication). The VIP:F-T cell line was derived from a primary culture of skin fibroblasts from an adult woman with a malignant melanoma (*19*). Morphologically transformed cells appeared in untreated 10-week old cultures, and these cells gave rise to

the VIP:F-T cell line. VIP:F-T cells are fibroblastic rather than melanocytic. It was not possible to verify the origin of this cell line using molecular analysis, since the parental cell line was not available (*16*).

Assay for Tumorigenicity in Nude Mice

BALB/c athymic nude mice from a closed colony were utilized as tumor hosts. Serum from heterozygous female breeders was tested regularly by Microbiological Associates (Rockville, MD), and was consistently free of antibodies to K virus, polyoma virus, murine hepatitis virus, ectromelia virus, GD VII virus, murine adenovirus, lymphocytic choriomeningitis virus, minute virus of mice, Sendai virus, pneumonia virus of mice, reovirus type 3, EDIM virus, murine cytomegalovirus, Car bacillus, and Mycoplasma pulmonis. Mice were maintained in accordance with Michigan State University guidelines in isolators illuminated by fluorescent light, with a 12 hour/12 hour light/dark cycle, temperature of 70-75°F, and humidity of 30-50%. They were housed in plastic cages at up to five mice per cage. Cages had pine shaving bedding and individual water bottles. Mice were fed pelleted laboratory chow.

Mice of both sexes were used as tumor hosts. From 1×10^6 to 5×10^6 sarcoma-derived or spontaneously-transformed human fibroblasts were injected into the subcutis on the dorsal midline of 6-10 week old mice. Afterwards, mice were examined weekly for tumor development. Mice were killed by CO_2 inhalation when tumors were 2-3 cm in diameter, or sooner if the tumor became ulcerated or the mouse showed signs of illness such as listlessness or weight loss. If no tumor developed within 8 months of injection, the cell line was considered non-tumorigenic.

Collection and Processing of Tissues

Mice received a complete necropsy that included gross and microscopic examination of the tumor itself, axillary, cervical, and mesenteric lymph nodes, thyroid gland, parathyroid glands, adrenal glands, salivary glands, trachea, lungs, heart, liver, spleen, pancreas, and kidneys. Specimens for routine microscopic examination were fixed in neutral buffered 10% formalin. Sections of tumor for immunoperoxidase staining were fixed in freshly-prepared B-5 fixative for 2 hours, then transferred to 70% ethanol.

Tissues were processed by routine histologic methods and imbedded in paraffin blocks. Tumors were serially sectioned into 2-3 mm thick slices and the entire tumor was imbedded, so that 3-10 tumor sections representing different areas were examined microscopically. Six micron sections were cut and stained with hematoxylin and eosin for general examination. Additional sections were cut and stained with Gomori's trichrome stain or silver stain to demonstrate thick and thin collagen fibers. In some cases, frozen sections of formalin-fixed tissue were prepared and stained for lipid with Oil Red O.

Additional sections of B-5-fixed tissue were cut and stained by an immunoperoxidase method for vimentin, desmin, S-100, alpha-1-antichymotrypsin, and Factor VIII antigen. Slides were stained in batches of 20, each batch including 18 tumors, a positive human tissue control, and a negative antibody control. Briefly, 3 micron sections were placed on poly-L-lysine coated slides and incubated overnight at 60°C. Slides were de-Zenkerized, rinsed, and immersed in methanol for 10 minutes. Endogenous peroxidase activity was blocked by incubation for 10 minutes in H_2O_2, followed by 5 minutes in normal goat serum. Slides were then labelled with one of five primary antibodies: monoclonal mouse anti-vimentin, monoclonal mouse anti-desmin, polyclonal rabbit anti-S-100, polyclonal rabbit anti-alpha-1-antichymotrypsin, and polyclonal rabbit anti-Factor VIII (all from Lipshaw, Detroit, MI). After treatment with the appropriate linking antibody and labelling reagent, slides were

exposed to chromogenic substrate for 5-20 minutes. Slides were counterstained with Mayer's hematoxylin.

RESULTS

General Observations

The results of the assay of the cell lines for tumorigencity are summarized in Table 2. The SW-982 cell line did not produce tumors in any mice injected. This cell line also failed to produce tumors in previous experiments in our laboratory (*20*). The SW-684 cell line produced a tumor in only one of three mice. The other cell lines produced tumors in all mice injected. The microscopic characteristics of these tumors are described below and summarized in Table 3.

Tumors grew as nodular masses within the subcutis. Microscopically, they were not encapsulated and did not compress surrounding tissues as they grew. Instead, enlarging tumors invaded adjacent adipose tissue and skeletal muscle as they grew, entrapping individual adipocytes and muscle fibers within the tumor mass. Tumors did not invade bone or cartilage, nor penetrate through the thoracic wall in any mouse, although this may reflect the relatively early stage in tumor growth at which mice were killed rather than an inability of tumors to invade these tissues. Spontaneous metastases were not found in any tumor-bearing mouse. This also may reflect the relatively short time the tumors were allowed to grow.

We stained tumors produced by six of the seven cell lines using an immunoperoxidase method and, as expected, all tumors contained cells that stained positively for vimentin intermediate filaments (Table 4). In fact, such staining was taken to indicate that processing and fixation had been sufficient to preserve tissue antigens. Positive staining for vimentin also gave evidence that the tumors were composed of human cells rather than mouse cells transformed in vivo, because the monoclonal antibody used was specific for an epitope on human vimentin filaments.

SHAC Cells

The SHAC cell line formed densely cellular tumors consisting of short interwoven bundles of cells that occasionally intersected in a manner reminiscent of a storiform pattern (Figure 1). Focal necrosis was not prominent, although apoptosis was regularly present. Trichrome and reticulin stains revealed that collagen fibers were scarce. Fibers tended to be thin and usually were located at the periphery of individual fascicles in association with stromal blood vessels, suggesting that they were the product of host cells rather than tumor cells. Tumors cells were closely packed with very little cytoplasm and indistinct cell borders (Figure 2). Nuclei were moderately pleomorphic but generally oval with evenly dispersed, finely granular chromatin and 1 or 2 small nucleoli that were visualized best in B-5-fixed tissue. Mitoses were moderately common (3-5/hpf). Tumors produced by SHAC cells contained scattered cells that stained positively for desmin (Figure 3). Desmin-positive cells had diffuse cytoplasmic staining that was slightly less intense than that of mouse skeletal muscle fibers and vascular smooth muscle cells in the same section.

HT1080 Cells

The HT1080 cell line formed tumors consisting of solid sheets of closely packed but distinct cells with a scant fibrovascular stroma (Figure 4). Focal necrosis was only occasionally present. At times the tumor cells were segregated into discrete small clusters by the stroma. This arrangement, coupled with the epithelioid appearance of the cells, made the tumors resemble carcinomas rather than sarcomas. Fascicle formation was never observed. Trichrome and reticulin stains revealed that collagen fibers were scarce and always associated with stromal elements; there was no evidence of collagen production by tumor cells. Tumor cells were very large with a moderate amount of basophilic cytoplasm (Figure 5). They were generally round or polyhedral and usually had distinct cell borders.

Each cell contained a single, centrally-located nucleus. Nuclei were somewhat pleomorphic but tended to be oval or indented (reniform). They contained a granular chromatin that was concentrated along the nuclear membrane, making it easy to see the 3 to 5 nucleoli within each nucleus. Mitoses were very common (5-10/hpf).

8387 Cells

The 8387 cell line formed tumors that consisted of short interwoven bands of cells intersecting at irregular angles that sometimes produced a storiform pattern (Figure 6). Multifocal necrosis was a consistent feature of these tumors, and apoptosis was also common. Thick and thin collagen fibers were common and surrounded virtually every tumor cell. Tumor cells were spindle shaped or crescentic with fibrillar basophilic cytoplasm and cell borders that were fairly distinct against the eosinophilic collagenous matrix (Figure 7). Nuclei were large and moderately pleomorphic. They were generally round or oval with a finely granular chromatin that, while evenly dispersed, did not obscure the 1 to 3 large magenta nucleoli that each nucleus contained. The mitotic rate of these tumors was low (1-3/hpf).

HuT-14 Cells

As expected, the HuT-14 subline of the 8387 cell line produced tumors very similar to those of 8387 cells. The HUT-14 cell line produced tumors consisting of interwoven bundles of spindle cells that sometimes formed a storiform pattern (Figure 8). In contrast to tumors produced by 8387 cells, necrosis was not a common feature of tumors produced by HuT-14 cells. Also in contrast to tumors produced by 8387 cells, tumors produced by HUT-14 cells frequently had areas where cells were somewhat loosely arranged within a pale staining, foamy, myxoid matrix. Oil Red O staining for fat revealed only occasional

small lipid droplets within tumor cells; no signet ring cells or physaliferous cells were found. Collagen fibers were common in these tumors as they were in tumors formed by 8387 cells, and almost every tumor cell was surrounded by collagen. Tumor cells were spindle shaped with fibrillar cytoplasm (Figure 9). Nuclei were moderately pleomorphic, ranging from oval to elongated with a finely granular chromatin and 1 to 3 small nucleoli. Mitoses were not common (1-3/hpf).The tumors formed by HUT-14 cells stained positively for S-100 antigen (Figure 10). Staining was diffuse and cytoplasmic, but slightly less intense than that of mouse peripheral nerve fibers within the same section.

NCI Cells

The NCI cell line formed tumors consisting of interwoven fascicles of spindle cells (Figure 11). At low magnification the tumors resembled those produced by 8387/HUT-14 cells, but the cells that comprised NCI tumors were more pleomorphic. Focal necrosis was not common, but apoptosis was. Collagen fibers were common throughout the tumors. Tumor cells were variable in size and shape but tended to be spindle shaped with scant, fibrillar, eosinophilic cytoplasm (Figure 12). Nuclear pleomorphism was great. Nuclei varied from round with multiple indentations to oval or elongated. Chromatin was coarsely granular and clumped along the nuclear membrane. Nucleoli were large, multiple, and often bizarrely-shaped. Mitoses were not common (1-3/hpf).

SW-684 Cells

SW-684 cells produced a tumor in only one of three mice injected. In that mouse, the tumor consisted of cells arranged in short, interwoven fascicles mixed with solid sheets. In many areas the cells were separated into clusters by a delicate fibrovascular stroma. There were extensive coalescing areas of necrosis within the tumor (Figure 13). Cells were

large and extremely pleomorphic (Figure 14). They varied from round or oval to polyhedral in shape, with granular basophilic cytoplasm and a single large nucleus. Nuclei were round or oval, sometimes reniform, with coarsely clumped chromatin located mostly along the nuclear membrane. There were 1 to 3 very large nucleoli in each nucleus. Mononuclear tumor giant cells were easy to find, and mitotic figures were moderately common (3-5/hpf).

VIP:F-T Cells

Of all cell lines tested, VIP:F-T cells formed tumors that most closely resembled human fibrosarcomas. Tumors consisted of long interwoven fascicles of large spindle cells that often intersected at regular angles to form a herringbone pattern (Figure 15). Multifocal necrosis was a prominent and consistent feature of these tumors. Trichrome and silver staining revealed that thick collagen fibers were common throughout the tumor and were associated with nearly every tumor cell. Thin collagen fibers were scarce and confined to the stroma. The cells had a small amount of fibrillar eosinophilic cytoplasm (Figure 16). Individual cell borders were difficult to discern on HE-stained sections because the cytoplasm could not be distinguished easily from the collagenous matrix between cells. However, on trichrome-stained sections the elongated shape of the cells was easy to see. Nuclei were also elongated and moderately pleomorphic; many had the blunt ends typical of leiomyosarcoma cells instead of the tapered ends usually associated with fibrosarcoma cells. Chromatin was confined to the nuclear membrane giving the nuclei a vesicular appearance. Narrow bands of chromatin often bridged the width of the nucleus or connected nucleoli to the nuclear membrane. Nucleoli were generally small and multiple. Mitoses were very common (5-10/hpf).

DISCUSSION

The tumors produced by the sarcoma-derived cell lines and the spontaneously-transformed cell line varied in their microscopic features but, with the exception of those produced by HT1080 cells, were partly or entirely spindle cell sarcomas with cells arranged in interwoven bands. On the other hand, the microscopic features of the tumors produced by each particular cell line were distinctive, consistent, and reproducible, so that it was easy to recognize which cell line had produced a particular tumor even without prior knowledge of which cell line had been injected. Such phenotypic variety among tumors produced by these cell lines is not unexpected. Fogh and Trempe (*21*) pointed out over a decade ago that cell lines derived from the same type of tumor, based on pathologists' diagnoses, may have different characteristics, and that as a consequence, studies of many cell lines derived from tumors of similar histopathologic appearance was the best approach to investigating carcinogenesis. There are discrepancies in some of the published reports of the derivation of the sarcoma-derived cell lines used in the studies reported here. In view of the conflicting reports regarding the histopathologic diagnosis of the original tumor from which the HT1080 and 8387 cell lines were derived, and whether it was primary or metastatic, and current ambiguities in the classification of soft tissue sarcomas (discussed in *22-26*) it is not surprising that cell lines supposedly derived from the same type of tumor differ in many characteristics, including the type of tumor they produce.

Transformation of mouse cells in association with injected human tumor cells has been reported many times (*27-35*) and is a potential complication of assays for tumorigenicity in nude mice. Evidence that the tumors we studied were composed of human cells rather than mouse cells comes from our finding that they stained with antibody specific for human vimentin intermediate filaments.

The tumors were not expected to stain positively for desmin intermediate filaments or S-100 protein. Desmin appears to be expressed only in normal muscle tissue and in tumors derived from such cells, and its presence is routinely utilized by pathologists to distinguish muscle cell tumors from other types of sarcomas (*36, 37*). However, there have been rare reports of fibrosarcomas that stain positively for desmin (*38, 39*), and with the electron microscope myofilaments can be seen within cells of some fibrosarcomas (*40*), prompting investigators to dub such tumors myofibroblastomas (*41*). Recently, Skalli et al. (*42*) demonstrated that the semimalignant proliferative lesions of fibromatosis contain myofibroblasts that have both vimentin and desmin intermediate filaments. Furthermore, the SHAC cell line that produced tumors containing desmin-positive cells was derived from a gastric sarcoma, and smooth muscle tumors are the most common type of sarcoma in the human gastrointestinal tract (*24*). The expression of desmin intermediate filaments by the SHAC cell line may reflect a general tendency of gastrointestinal sarcomas toward myogenic, or at least myofibroblastic, differentiation.

The positive staining for S-100 of tumors produced by Hut-14 cells is more difficult to explain. The 8387 cell line from which this subline was derived produced tumors that were S-100-negative. Clonally-derived populations are known to differ from their parent cell line and from each other in many characteristics, including growth characteristics in vivo (*43, 44*). The fact that virtually every cell within the tumors produced by HuT-14 cells expressed S-100 protein is consistent with the hypothesis that the HuT-14 cell line was derived from a single variant cell with an activated S-100 gene. Although the HuT-14 and 8387 cell lines produced tumors that were quite similar, necrosis was prominent in tumors formed by 8387 cells but almost never seen in tumors formed by HuT-14 cells. Also, tumors produced by HuT-14 cells grew much more rapidly than tumors produced by 8387 cells, suggesting that the two cell lines differed in other properties besides S-100 expression. S-100 protein is expressed in a limited number of normal tissues and tumor types. Among

spindle cell tumors, those exhibiting chondrocytic, neurogenic, or melanocytic differentiation are generally S-100-positive, while fibrosarcomas are S-100-negative (*45, 46*). Tumors produced by HuT-14 cells showed no evidence of cartilage matrix production by light microscopy and no evidence of Schwann cell or melanocytic differentiation by electron microscopy (unpublished observations), so the activation of S-100 did not appear to be part of a general program of non-fibroblast differentiation. More likely, S-100 expression resulted from activation of a single gene in this subline.

The tumors produced by the sarcoma-derived cell lines and the spontaneously-transformed fibroblast line resembled those produced by human fibroblasts transformed in vitro by ras oncogene transfection (*4*) in latency period, cytologic features, morphologic patterns of growth, and failure to mestastasize spontaneously from the subcutis. In addition, comparison of the tumors with each other and with published descriptions of spontaneous human soft tissue sarcomas revealed that:

a) The HT1080 cell line, whose cells contain an N-ras oncogene (*5*), made round cell sarcomas that closely resembled those produced by one line of MSU-1.1 fibroblasts transformed by an N-ras oncogene derived from the human leukemia cell line 8402. These tumors did not resemble fibroblastic tumors that arise spontaneously in humans.

b) The SHAC cell line, whose cells contain an N-ras oncogene and an amplified c-myc gene (*6*), made spindle cell sarcomas in which some of the cells contained desmin intermediate filaments. Desmin-positive spindle cell sarcomas were also produced by two strains of N-ras-transformed MSU-1.1 fibroblasts. MSU-1.1 fibroblasts contain a v-myc oncogene, so N-ras transformants have alterations in the same two oncogenes that are altered

in SHAC cells. These tumors resembled myofibroblastomas that arise spontaneously in humans.

c) The HuT-14 subline made spindle cell sarcomas that were S-100-positive, and so did one line of MSU-1.1 fibroblasts transformed by the H-ras oncogene derived from the human bladder carcinoma cell line T24. These tumors resembled neurofibrosarcomas that arise spontaneously in humans.

d) The VIP-F:T cell line made spindle cell sarcomas with prominent herringbone patterns and abundant collagen fibers, and these tumors resembled those produced by several lines of MSU-1.1 fibroblasts transformed by the H-ras and N-ras oncogenes. These tumors resembled fibrosarcomas that arise spontaneously in humans.

e) The SW-684 cell line made a pleomorphic sarcoma with mononuclear giant cells that resembled those produced by KMST-6 fibroblasts or GM637 fibroblasts transformed by the T24 H-ras oncogene. These tumors resembled fibrosarcomas or malignant fibrous histiocytomas that arise spontaneously in humans.

f) No sarcoma-derived or spontaneously-transformed cell line produced myxoid sarcomas similar to those produced by several lines of K-ras-transformed MSU-1.1 fibroblasts, pleomorphic sarcomas with multinucleated giant cells similar to those produced by one line of N-ras-transformed MSU-1.1 fibroblasts, or spindle cell sarcomas with a whorling pattern similar to those produced by one line of H-ras-transformed MSU-1.1 fibroblasts.

These studies reveal that the range of tumor types produced by ras-transformed fibroblasts in previous studies (*4*) was not an artifact of their in vitro creation. Cell lines

derived from human sarcomas or spontaneously-transformed in vitro also produced a range of tumor types, including sarcomas that expressed desmin or S-100, supporting the idea that in vitro transformation by ras oncogene transfection is a relevant model for in vivo carcinogenesis.

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Table 1. Origin of Sarcoma-derived and Spontaneously-transformed Cell Lines

Cell Line	Derivation	References
SHAC	gastric fibrosarcoma	6
HT1080	fibrosarcoma (primary or metastatic?) in the hip	7,8
8387	fibrosarcoma (osteosarcoma?)	9,10,11,12
HuT-14	subline of 8387	13,14,15,17
SW-684	recurrent fibrosarcoma--site unspecified	18
SW-982	axillary fibrosarcoma	12
NCI	fibrosarcoma--site unspecified	C. Cooper, personal communication
VIP:F-T	skin fibroblast culture transformed in vitro	19

Table 2. Tumors Produced by Human Sarcoma-derived Cells or Spontaneously-transformed Human Fibroblasts

Cell Line	Tumors Produced/ Mice Injected	Mean Latency Period	Morphologic Classification
SHAC	5/5	20 days	Spindle cell sarcoma
HT1080	4/4	21 days	Round cell sarcoma
8387	5/5	88 days	Spindle cell sarcoma
HuT-14	3/3	28 days	Spindle cell sarcoma
NCI	3/4	63 days	Spindle cell sarcoma
SW-982	0/4	-----	-----
VIP:F-T	4/4	17 days	Spindle cell sarcoma

Table 3. Microscopic Features of Tumors Produced by Human Sarcoma-derived Cells or Spontaneously-transformed Human Fibroblasts

Cell Line	Predominant Cell Type	Arrangement of Cells	Collagen Fibers in Tumors	Necrosis in Tumors
SHAC	Spindle cells	Short fascicles, intersect at random	Scarce, stromal	Uncommon, single cells
HT1080	Epithelioid round cells	Solid sheets	Scarce, stromal	Uncommon, focal
8387	Spindle cells	Short fascicles, intersect at random	Abundant	Common, multifocal
HuT-14	Spindle cells	Short fascicles, intersect at random	Abundant	Uncommon, focal
NCI	Spindle cells	Short fascicles, intersect at random	Moderate	Uncommon, single cells
SW-684	Pleomorphic, giant cells	Pleomorphic, sheets and fascicles	Scarce, diffuse	Common, multifocal
VIP:F-T	Spindle cells	Broad fascicles, herringbone pattern	Abundant	Common, multifocal

Table 4. Immunoperoxidase Staining of Tumors Produced by Human Sarcoma-derived Cells and Spontaneously-transformed Human Fibroblasts[a]

Cell Line	Vimentin	Desmin	S-100	Alpha-1-anti chymotrypsin	Factor VIII antigen
SHAC	+	+[b]	-	-	-
HT1080	+	-	-	-	-
8387	+	-	-	-	-
HuT-14	+	-	+[c]	-	-
NCI	+	-	-	-	-
VIP:F-T	+	-	-	-	-

[a]Except for the SHAC line, two tumors of each type were stained, and 2-4 sections of each tumor were examined. Four tumors produced by SHAC cells were examined. Tumors produced by SW-684 cells were not examined.

[b]There was diffuse cytoplasmic staining of scattered tumor cells.

[c]There was diffuse cytoplasmic staining of most tumor cells.

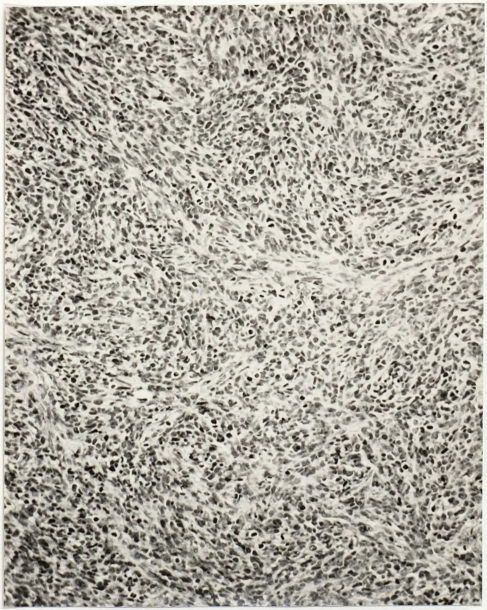

Figure 1. Sarcoma produced by SHAC cells. Cells are arranged in short interwoven fascicles (HE, 200x).

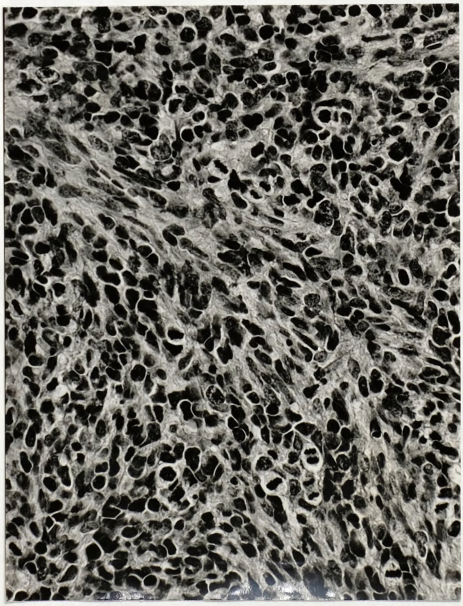

Figure 2. Sarcoma produced by SHAC cells. Closely packed cells with numerous mitotic figures (arrows) (B-5, 500x).

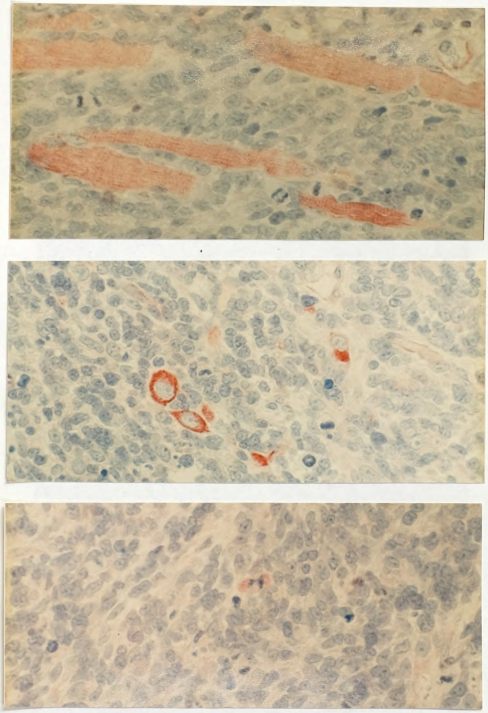

Figure 3. Sarcoma produced by SHAC cells stained by the immunoperoxidase method for desmin: a) Staining of murine skeletal muscle and arteriolar smooth muscle at periphery of tumor. Compare intensity of staining to that of tumor cells in b) and c). b) SHAC tumor with several strongly desmin-positive cells. c) SHAC tumor with mitosis of desmin-positive cell (all at 400x).

Figure 4. Sarcoma produced by HT1080 cells. Epithelioid cells arranged in solid sheet (HE, 200x).

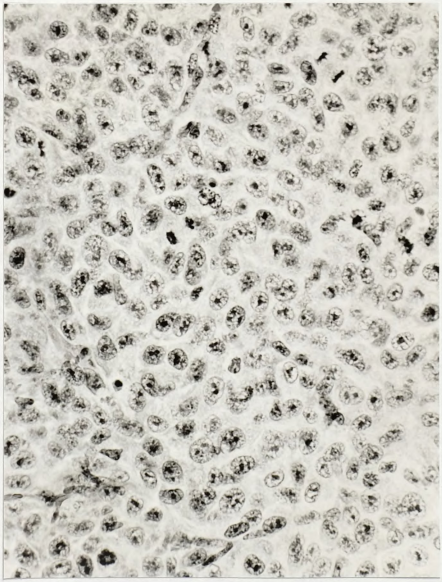

Figure 5. Sarcoma produced by HT1080 cells. Very large cells with oval and indented nuclei (HE, 500x).

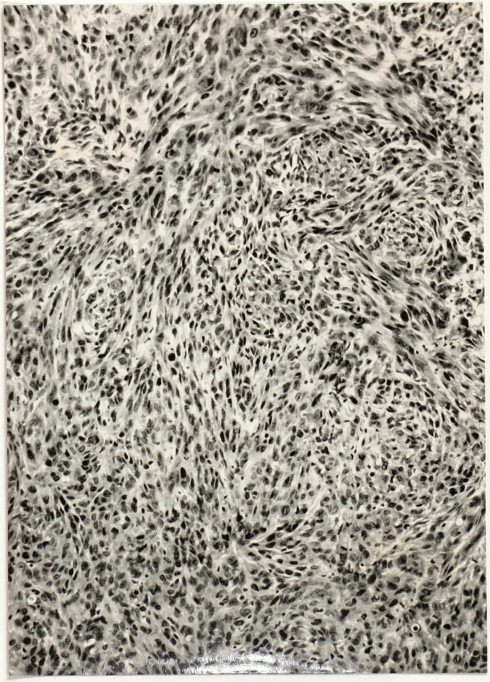

Figure 6. Sarcoma produced by 8387 cells. Short interwoven bands of cells (B-5, 200x).

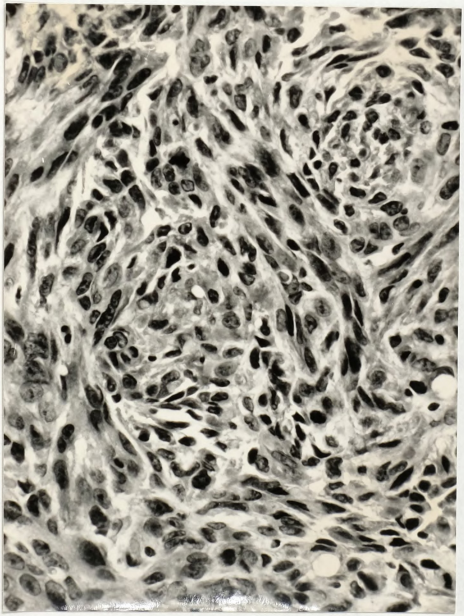

Figure 7. Sarcoma produced by 8387 cells. Moderately pleomorphic spindle cells (B-5, 500x).

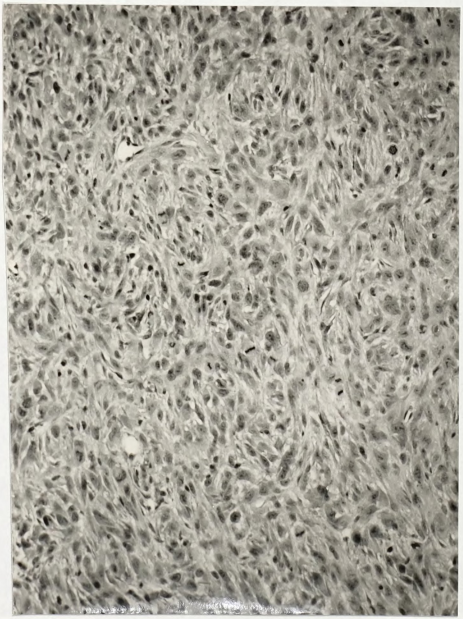

Figure 8. Sarcoma produced by HuT-14 cells. Cells arranged in short interwoven bands. Compare with Figure 6 (HE, 200x).

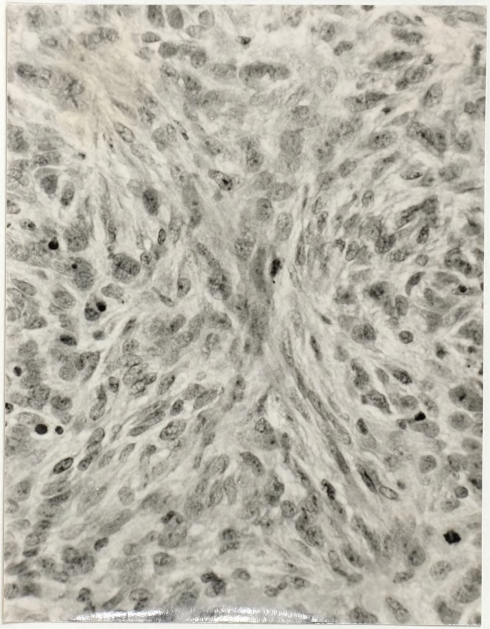

Figure 9. Sarcoma produced by HuT-14 cells. Note similarity between this tumor and Figure 7 (HE, 500x).

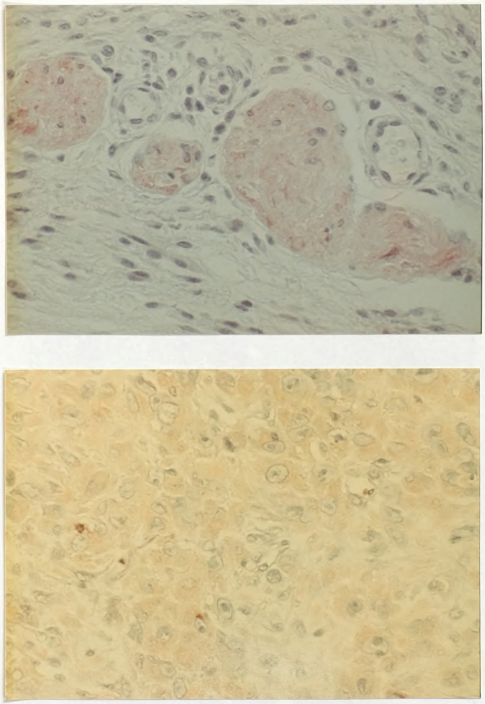

Figure 10. Sarcoma formed by HuT-14 cells stained by the immunoperoxidase method for S-100: a) Murine peripheral nerve. Compare intensity of S-100-positive reaction to that of tumor cells in b). b) Tumor with diffuse cytoplasmic staining of most cells (both at 400x).

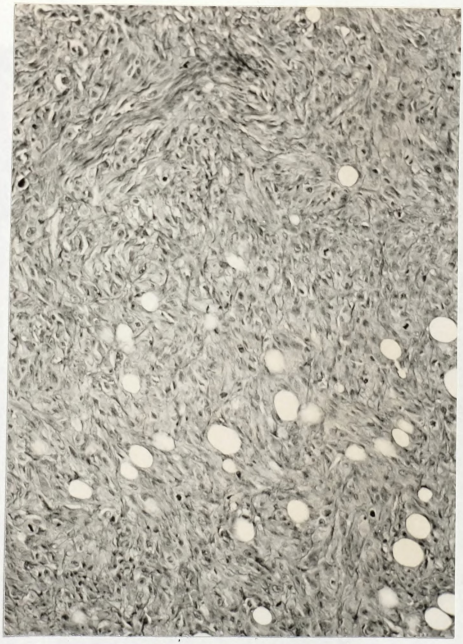

Figure 11. Sarcoma produced by NCI cells. Thick collagen fibers and entrapped adipocytes are prominent (HE, 200x).

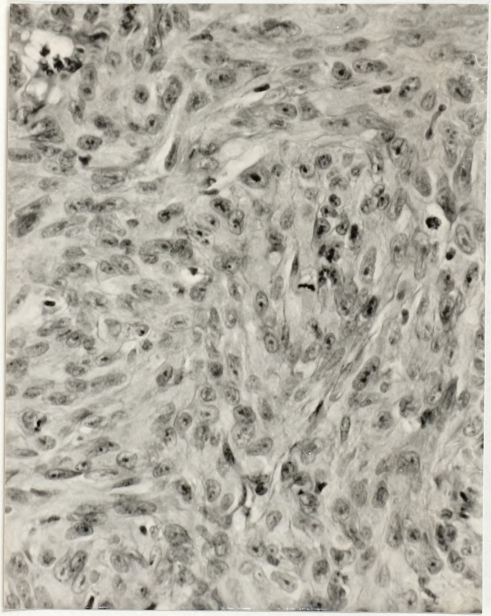

Figure 12. Sarcoma produced by NCI cells. Pleomorphic spindle cells (HE, 500x).

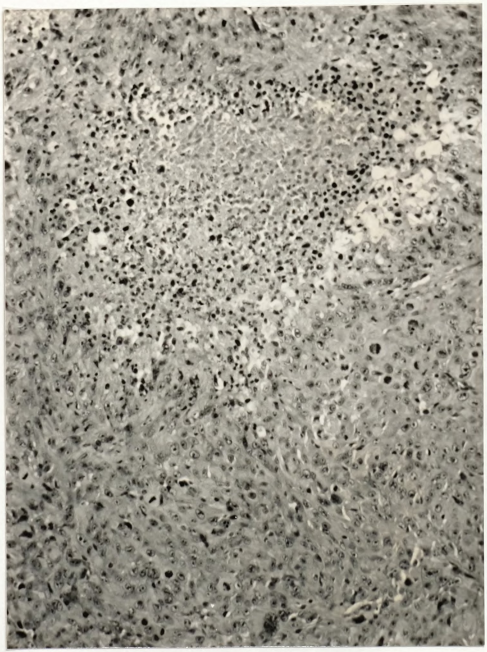

Figure 13. Sarcoma produced by SW-684 cells. Large area of necrosis in the tumor (HE, 200x).

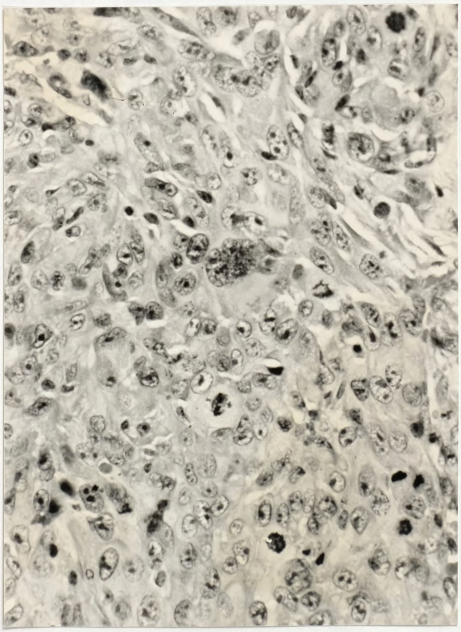

Figure 14. Sarcoma produced by SW-684 cells. Large and pleomorphic cells, with tumor giant cell (arrow) (HE, 500x).

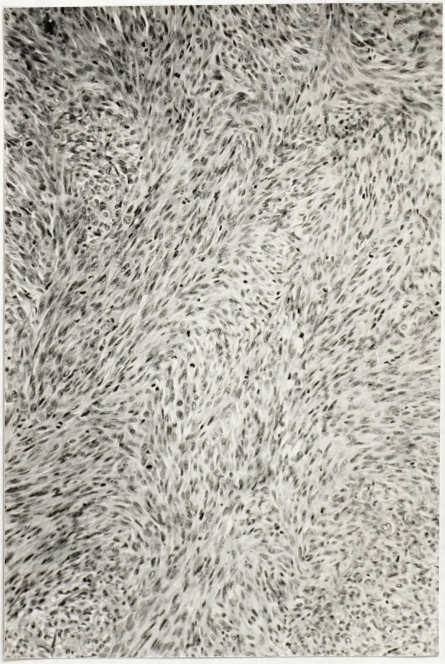

Figure 15. Sarcoma produced by VIP:F-T cells. Broad bands of cells in a herringbone pattern (HE, 200x).

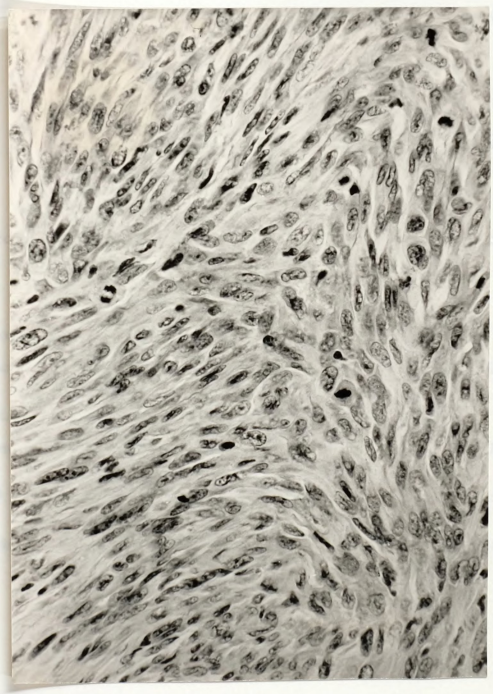

Figure 16. Sarcoma produced by VIP:F-T cells. Spindle cells with elongated nuclei (HE, 500x).

EXPERIMENTAL METASTASIS OF *RAS*-TRANSFORMED HUMAN FIBROBLASTS

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Running Title: **Experimental Metastasis**

ABSTRACT

Cell strains derived from infinite life span human fibroblasts transformed in vitro by transfection with a K-ras, H-ras, or N-ras oncogene were injected intravenously and intraperitoneally into athymic nude mice to see if ras oncogenes influence the metastatic capacity of malignantly transformed human fibroblasts. The ras-transformed cells metastasized via the bloodstream or by implantation in the peritoneal cavity. Metastasis was influenced by the transfected ras oncogene and the recipient cell line. Metastatic tumors often arose in skeletal muscle, but this was because ras-transformed cells spread from the injection site via lymphatics rather than because muscle was a more favorable tissue for tumor formation by ras-transformed cells. The microscopic features of metastatic tumors almost always resembled those of primary tumors produced by the same ras-transformed cell strains. These results confirm that ras oncogenes can transform non-tumorigenic infinite life span human fibroblasts into tumorigenic cells capable of experimental metastasis, and provide direct evidence that ras oncogenes may play a role in the metastatic process in human cancer.

Key Words: **H-ras, K-ras, N-ras, metastasis, fibroblast**

INTRODUCTION

Oncogenes of the ras family are capable of malignantly transforming rodent fibroblasts, and McCormick and his colleagues recently succeeded in malignantly transforming infinite life span human fibroblasts with K-ras, N-ras, or H-ras oncogenes (11,15,33). The resultant cell strains produced progressively-growing, invasive sarcomas with a variety of microscopic appearances when injected subcutaneously into athymic nude mice (7). These sarcomas were characterized by cytologic features, patterns of growth, and a panel of immunoperoxidase stains, and found to be similar in many respects to tumors produced by human sarcoma-derived cell lines and a spontaneously-transformed human fibroblast line (8).

Transformation of rodent fibroblasts by ras oncogenes has been reported to confer metastatic ability on cells, as judged by both spontaneous metastasis in tumor-bearing mice and experimental metastasis, in which tumors form after intravenous inoculation of transformed cells (2,3,4,5,6,9,10,13,18,25,29,30,32,34). In prior studies with ras-transformed human cell strains, spontaneous metastasis from primary subcutaneous tumors was never observed (7). Whether this reflected an inability of these tumorigenic cells to metastasize or simply a lack of sufficient time for spontaneous metastasis to occur was unknown, since tumors appeared soon after injection and grew rapidly so that tumor-bearing mice seldom survived longer than 4 weeks. To determine if ras-transformed human fibroblasts could metastasize, cells were injected into the bloodstream or peritoneal cavity of athymic nude mice. To test whether or not the transfected ras-oncogene influenced experimental metastasis, we compared metastatic tumor formation by cell strains derived from a single receipient fibroblast line transformed by three different ras oncogenes. To test whether or not the nature of the recipient cell line influenced experimental metastasis, we compared

metastatic tumor formation by cell strains derived from three different fibroblast lines transformed by transfection with the same H-ras oncogene. To investigate whether or not the microscopic features of metastatic tumors would resemble those of subcutaneous tumors produced by the same cell strain, we compared metastatic tumors to primary subcutaneous tumors produced by ras-transformed cell strains in this study and previous studies (7). We found that ras oncogenes transformed infinite life span human fibroblasts not only into tumorigenic cells, but also into fully malignant tumor cells capable of experimental metastasis via the circulation or implantation within body cavities. The transfected oncogene and the nature of the recipient cell line both influenced metastasis.

EXPERIMENTAL DESIGN

Cell lines used in these studies are listed in Table 1. In initial experiments we evaluated the metastatic ability of ras-transformed fibroblast strains by injecting cells into the tail vein or peritoneal cavity of mice. As controls, we also injected mice with spontaneously-transformed human fibroblasts (cell line L55I-3T) and with cells from the human sarcoma-derived cell line HT1080. Besides injecting transformed cells intravenously we also injected them subcutaneously in additional mice to confirm that the cells were viable and tumorigenic. After being injected with transformed cells, mice were examined weekly and maintained for six months or until metastatic tumors developed. All mice received complete necropsies that included microscopic examination of major organs for metastatic tumors.

To investigate the influence of ras oncogenes on metastasis, we compared metastasic tumor formation by the various ras-transformed MSU-1.1 fibroblast strains. To investigate the influence of the recipient cell line on metastasis, we compared metastatic tumor formation by three cell strains derived from three different infinite life span fibroblast lines transformed by the same H-ras oncogene. To investigate factors affecting the distribution of metastatic tumors, a single ras-transformed cell strain (N-ras-3T) was injected intracardially, intravenously, intramuscularly, and subcutaneously.

RESULTS AND DISCUSSION

General Observations

All of the cell lines and cell strains tested produced tumors when injected subcutaneously or intraperitoneally, indicating that the inocula contained viable tumorigenic cells. Mice that received intraperitoneal inoculations developed multiple tumors within their abdominal cavities. Abdominal tumors not only grew as nodular masses on the parietal and visceral peritoneum (Figure 1), but also invaded underlying tissues in many mice (Figure 2).

The number of mice that developed metastatic tumors following intravenous inoculations are shown in Tables 2 and 3. All three ras oncogenes could confer upon infinite life span human fibroblasts the prime attribute of cancer cells, the ability to metastasize. Metastatic tumors developed at various sites, and we were uncertain at first whether we could compare the rate of tumor growth at these different sites because our endpoint for subcutaneous or intramuscular tumors was a diameter of 1 cm, but our endpoint for intrathoracic or intraabdominal tumors was debilitation or palpable tumor. In fact, these two endpoints turned out to be very similar, since by the time metastatic tumors in the abdomen or thorax reached 1 cm in diameter they either caused obvious dyspnea or were palpable through the body wall, and metastatic tumors that arose in skeletal muscles could be monitored directly.

Effect of Transfected ras Oncogene on Metastasis

The ras oncogenes differed in their ability to confer metastatic properties upon the MSU-1.1 fibroblast line (Table 2). One of the mice injected with the N-ras-3T cell strain was killed 45 days after injection because it was losing weight. At necropsy we found a

severe malocclusion with overgrowth of incisors that probably prevented the mouse from eating and accounted for the weight loss. No tumors were found in any tissues from this mouse, but the rest of the mice that received N-ras-3T cells intravenously developed metastatic tumors, giving an overall frequency of metastasis of 90%. Results were similar with the sarcoma-derived cell line, HT1080, which carries an N-ras oncogene (14). One mouse that received HT1080 cells developed a bacterial otitis media and when we killed it 56 days after injection we found no tumors; however, the remaining mice that received HT1080 cells developed metastatic tumors. A smaller percentage of mice receiving N-ras-5T cells (50%) developed tumors. Only one of the two H-ras-transformed cell strains (H-ras-2T) formed metastatic tumors at all, and it did so with a frequency of 40%. Although both K-ras-transformed cell strains formed metastatic tumors, they did so with low frequencies (10% and 30%). The spontaneously-transformed MSU-1.1 fibroblast line, L55I-3T, also had a low frequency of experimental metastasis (27%).

The ras-transformed cell strains and control cell lines fell into 2 groups with respect to the time required for metastatic tumors to form following intravenous inoculation. One N-ras-transformed cell strain (N-ras-3T), the spontaneously-transformed fibroblast line L55I-3T, and sarcoma-derived cell line HT1080 formed metastatic tumors in 9-12 weeks. The other N-ras-transformed cell strain (N-ras-5T), both K-ras-transformed cell strains, and the metastatic H-ras-transformed cell strain (H-ras-2T) took 17-20 weeks to form metastatic tumors.

The relationship between ras oncogenes and metastasis has been the subject of numerous investigations in recent years (16,17) and was recently reviewed by Nicolson (23). Most studies have involved transfection of ras oncogenes into rodent fibroblasts. Transfection of H-ras or N-ras oncogenes into mouse NIH 3T3 cells, mouse C3H 10T1/2 cells, rat fibroblasts, or Chinese hamster lung fibroblasts makes the cells capable of

spontaneous and experimental metastasis (2,3,4,6,9,10,18,25,28,30,32,34). Interpretation of these rodent cell transfection experiments is difficult, however, because often even untransfected or mock-transfected mouse and rat cells prove capable of spontaneous and experimental metastasis (6,10,12,25,31). We could find only one report of a metastasis study using ras-transfected human cells. Agnor et al. (1) transfected a cellular or viral H-ras oncogene into human breast carcinoma cells and found that ras had no effect on the frequency of spontaneous metastasis. Our studies extend the observations made in rodent cell systems to human cells, providing the first direct evidence that ras oncogenes may play a role in the metastatic process in human cancer.

Our results suggest that the ability of ras oncogenes to induce a metastatic phenotype is influenced by several factors, one of which is the transforming gene itself. For example, although N-ras and K-ras oncogenes each made MSU-1.1 fibroblasts metastatic, they were not equally efficient at doing so. These two oncogenes differ in at least two ways. First, the activating mutations are different: the 8402 N-ras oncogene has a single activating mutation in codon 12, while the K-ras oncogene has two such mutations in codons 13 and 59. Second, although the two genes belong to the same gene family they have somewhat different sequences and presumably have different normal physiologic functions. Investigation of the relationship between specific ras oncogenes and metastasis was complicated by the fact that each of the ras oncogenes used to transform MSU-1.1 fibroblasts was introduced in a different plasmid construction. Attempts to construct a series of plasmids that are identical except for the oncogene they carry are currently underway.

Effect of Recipient Cell Line on Metastasis

The nature of the parent cell line influenced acquisition of metastatic ability, as illustrated by comparing metastatic tumor formation by the H-ras-transformed MSU-1.1, KMST-6, and GM637 fibroblasts (Table 3). Like the H-ras-3T cell strain, H-ras-transformed GM637 fibroblasts failed to form metastatic tumors when injected intravenously. However, 7 of 9 mice injected intravenously with H-ras-transformed KMST-6 fibroblasts did develop metastatic tumors. An eighth mouse injected with H-ras-transformed KMST-6 fibroblasts developed signs suggestive of pulmonary metastasis but died unexpectedly on day 55 and was too autolyzed to be necropsied. Metastatic tumors produced by H-ras-transformed KMST-6 fibroblasts developed rapidly, with an average latency period of 65 days.

Muschel et al. (18) got similar results following transfection of infinite life span mouse fibroblast lines with v-H-ras. They found that H-ras-transformed NIH 3T3 cells made experimental metastases in nude mice but H-ras-transformed C127 cells did not.

Effect of Other Factors on Metastasis

Differences in the frequency of metastasis by the two N-ras-transformed MSU-1.1 cell strains or the two H-ras-transformed MSU-1.1 cell strains suggested that factors such as the site of integration of the transfected ras oncogene might have influenced metastasis. Integration site could directly affect the expression of the transfected oncogene itself, of adjacent normal cellular genes, or both. Host factors such as age, sex, nutritional status, health status, and hormonal status also could influence metastasis of ras-transformed cells. The role of host factors in experimental metastasis assays was recently discussed by Nowell (24) and Mareel and Van Roy (17). In addition, the particular method used to test for experimental metastasis can influence the results (17). Procedural differences such as

injection site, size of the inoculum, and environmental conditions under which mice are maintained can all affect experimental metastasis and make it difficult to compare studies.

Distribution of Metastatic Tumors

Table 4 lists the sites where metastatic tumors developed when cells were injected intravenously. Contrary to our expectations, few mice injected with the ras-transformed MSU-1.1 cell strains developed tumors in their lungs. Instead, tumors formed most often in the skeletal muscles of the thighs, pelvis, abdominal wall, and lumbar region. One mouse had a tumor in its dorsal thoracic cavity that was attached to the mediastinum but did not involve the lungs. Another mouse had tumors in its abdominal cavity and retroperitoneum, but the lungs were free of tumors. In contrast, the K1-T1/T cell strain and the HT1080 cell line chiefly formed pulmonary tumors when injected intravenously.

We considered at least two explanations for the unexpected distribution of metastatic tumors produced by the ras-transformed MSU-1.1 cell strains. On the one hand, ras-transformed cells injected into the tail vein may have traversed the pulmonary capillary bed, entered the systemic circulation, and escaped to form tumors at the various sites. In that case the predominance of intramuscular tumors reflected a predilection on the part of transformed cells to establish themselves in these sites. On the other hand, ras-transformed cells may have escaped from the tail vein at the injection site and entered the lymphatic system. Once there, cells could migrate along peripheral lymphatic vessels in an anterograde or retrograde fashion to form tumors in skeletal muscles of the caudal regions, or travel via the mesenteric and thoracic ducts to form tumors in abdominal organs, the retroperitoneum, and the dorsal thoracic cavity. In that case, the predominance of tumors in the skeletal muscles of the caudal regions simply reflected the pattern of lymphatic drainage from the injection site.

To distinguish between these possibilities, the original focus-derived cell strain N-ras-3 and the highly metastatic N-ras-3T line derived from it were injected by four routes. Intravenous and intracardial inoculations were done to see if tumor distribution was related to injection site or if tumors would develop in the same locations regardless of where we introduced cells into the bloodstream. Intramuscular (caudal thigh) and subcutaneous inoculations were done to see if tumors would develop more frequently or more rapidly at intramuscular sites, which would suggest that muscle tissue provided a more favorable environment for tumor growth.

Results of intracardial and intravenous injections appear in Table 5. Following intracardial inoculation of transformed cells, tumors developed most often within the thoracic wall, but also occurred in abdominal organs, on the peritoneum, and within muscles of the abdominal wall. None of the thoracic tumors involved the lungs. Tumors that occurred within abdominal muscles occurred in deep layers and projected as raised nodules on the peritoneum. One mouse had a small tumor on the endocardial surface of the left ventricle. In summary, the majority of tumors developed near the injection site following intracardial inoculation. The rest of the tumors developed where they would be expected to do so if the transformed cells had been deposited into the left ventricle. No tumors developed in skeletal muscles of the rear legs following intracardial inoculation.

When we injected the N-ras-3T cell strain into the tail vein in our first experiments, metastatic tumors formed in 9 of 10 mice but in our second series of experiments, metastatic tumors appeared in only 1 of 4 mice receiving the N-ras-3 cell strain and none of 5 mice that received N-ras-3T cells. The low frequency of metastasis in the second instance may be an artifact, since three of the four mice in the second series of experiments died within 35 days of being injected. The latency periods of N-ras-3T cells was 78 days in the first experiments and even longer in the second experiments.

The results of intramuscular and subcutaneous inoculations (Table 6) revealed that the N-ras-3 and N-ras-3T cell strains did not form tumors more rapidly or consistently in skeletal muscle than in the subcutis. Tumors developed over the same time period at both injection sites. At first glance it appeared that tumors developed more rapidly within muscle, since 1/15 as many transformed cells were injected intramuscularly as subcutaneously. However, this difference in initial cell numbers represented only 5-6 population doublings. These results were not consistent with the idea that muscle tissue offered a more favorable "soil" for tumor growth, but they did not rule out that transformed cells were targeted in some way to blood vessels within skeletal muscle.

These results suggested that tumors could develop not only from ras-transformed cells introduced into the bloodstream, but also from extravasated cells that either formed tumors locally (in the case of thoracic tumors) or travelled via lymphatics to form regional tumors at distant sites (in the case of skeletal muscle tumors). Metastatic tumors arising from transformed cells that escaped into lymphatic vessels at the time of inoculation necessarily must have arisen from much fewer cells than those arising from transformed cells injected into the bloodstream, suggesting that ras-transformed cells might actually metastasize more readily via lymphatic vessels than via the bloodstream. Why might this be so? One possibility is that ras-transformed cells survived better in the slow-moving lymphatic system than in the more turbulent vascular system. Another possibility is that cells carried to the lungs were rapidly removed by NK cells and pulmonary macrophages, while cells within extracellular spaces or lymphatic vessels were less rapidly or efficiently cleared by the immune system.

The apparent metastasis of extravasated transformed cells via lymphatics raises the question, why was such metastasis was never observed following subcutaneous or intramuscular inoculation, even though regional lymph nodes were examined routinely?

One possibility is that tumors developed too rapidly following subcutaneous inoculation to allow adequate time for spontaneous lymphatic metastasis to take place. This seems unlikely, since metastatic tumors developed nearly as rapidly following inoculations into the tail as primary tumors did following inoculations into the subcutis. A second possibility is that subcutaneous inoculation of a large number of ras-transformed cells incited a host response qualitatively or quantitatively different from that elicited by a smaller number of transformed cells injected intravenously, and that host defenses therefore inhibited lymphatic spread of transformed cells in the case of subcutaneous inoculation.

These studies point out a difference between most of the ras-transformed MSU-1.1 fibroblasts, which rarely formed lung tumors, and the N-ras-5T cell strain, the K1-T1/T cell strain, and the sarcoma-derived HT1080 cell line, which almost always formed lung tumors. The HT1080 cell line generally made single pulmonary tumors, while the ras-transformed cell strains produced multiple pulmonary tumors. We cannot explain why the HT1080 cell line produced only single metastatic lung tumors, although Alvarez and DeClerk (2) recently made the same observation.

Microscopic Appearance of Primary and Metastatic Tumors

Metastatic tumors were morphologically indistinguishable from primary subcutaneous tumors, with two exceptions. One exception was the K-ras-1aT cell strain, which formed myxoid sarcomas when given subcutaneously or intraperitoneally (Fig. 1), but non-myxoid spindle cell sarcomas when given intravenously (Fig. 2). The other exception was the N-ras-3T cell strain, which formed only spindle cell sarcomas when inoculated intravenously (Fig. 3), intracardially, or into the peritoneal cavity (Fig. 4), but whose parent cell strain, N-ras-3 forms chiefly pleomorphic sarcomas with multinuclear giant cells when injected into the subcutis (7). The N-ras-3 cell strain also produces mixed tumors (malignant

mesenchymomas) and spindle cell sarcomas subcutaneously, and in previously-reported studies (7) we were able to clonally-derive substrains of N-ras-3 in vitro that formed only spindle cell sarcomas subcutaneously. A similar selection process may have occurred in vivo to give rise to the N-ras-3T cell strain.

These observations support the idea that the phenotype of transformed cells in vivo is the result of host/cell interplay rather than an inherent property of the transformed cells themselves. As already discussed regarding metastasic ability, host factors and assay conditions such as injection site influence expression of a variety of phenotypic characteristics of transformed cells, including the pattern of tumor growth. We reached a similar conclusion in previous studies with the ras-transformed cell strains (7).

METHODS

Cell Lines and Strains

The derivation of ras-transformed human cell strains by Fry et al. (11), Hurlin et al. (15), and Wilson et al. (33) has been described elsewhere. Briefly, infinite life span human fibroblasts were transfected with a plasmid containing the 8402 N-ras, T24 H-ras, or viral K-ras oncogene linked to a neomycin resistance gene. Stable ras transfectants of KMST-6 fibroblasts, which are derived from human embryo fibroblasts treated with ^{60}Co gamma radiation (19,22), and GM637 fibroblasts, which are derived from adult dermal fibroblasts infected with SV40 virus, were isolated by growth in selective medium containing the antibiotic G418. In the case of MSU-1.1 fibroblasts, which are derived from newborn foreskin fibroblasts transfected with a v-myc oncogene, a different selection procedure had to be used, because MSU-1.1 fibroblasts already contain a neomycin resistance gene. The ras-transfected MSU-1.1 cell strains were isolated as morphologically-transformed cells growing as foci on a cell monolayer or as anchorage independent colonies in soft agar.

MSU-1.1, GM637, and KMST-6 fibroblasts are non-tumorigenic in nude mice, but they can be malignantly transformed by transfection with an oncogene belonging to the ras family (11,15,20,21,33). Cells can be recovered from tumors by mincing the tumor and inoculating tissue into 75 mm tissue culture flasks containing McM medium (27) with G418. In these studies, both primary transfectants and tumor-derived ras-transformed cells were used. In addition, we used two cell lines as controls. One was the sarcoma-derived cell line HT1080, obtained from the American Type Culture Collection. Rasheed et al. (26) derived HT1080 cells from a fibrosarcoma that arose in a 35-year old man who later died of metastatic disease. HT1080 cells contain an N-ras oncogene (14). As another control, we used a spontaneously-transformed MSU-1.1 cell strain, L55I-3T.

Assays for Experimental Metastasis in Athymic Nude Mice

BALB/c athymic nude mice from a closed colony were used as recipients for transformed cells in all experiments. Serum from heterozygous female breeders was tested regularly by Microbiological Associates (Rockville, MD) and was consistently free of antibodies to K virus, polyoma virus, murine hepatitis virus, ectromelia virus, GD VII virus, murine adenovirus, lymphocytic choriomeningitis virus, minute virus of mice, Sendai virus, pneumonia virus of mice, reovirus type 3, EDIM virus, murine cytomegalovirus, Car bacillus, and Mycoplasma pulmonis. Mice were maintained in isolators illuminated by fluorescent light, with a 12 hour/12 hour light/dark cycle, temperature of 70-75°F, and humidity of 30-50%. They were housed in plastic cages at up to five mice per cage. Cages had pine shaving bedding and individual water bottles. Mice were fed pelleted laboratory chow.

We used 6- to 10-week old mice of both sexes in these experiments. For injection, we suspended cells in McM medium at a concentration of 10^6 cells/ml. Mice received 0.2-0.5 mls. (2-5 x 10^5 cells) by one of four routes. Intravenous injections were given into the tail vein of ether-anesthetized mice using a 25-gauge needle. Intracardiac injections were given by inserting a 25-gauge needle through the left lateral thorax of anesthetized mice and aspirating slightly to check for blood to insure that the needle tip was within a heart chamber. Intramuscular injections were given into caudal thigh muscles, and intraperitoneal injections were given by inserting the needle on the ventral abdominal midline. Subcutaneous injections consisted of 2-5 x 10^6 cells injected between the shoulder blades.

We observed mice biweekly for signs of tumor development, and killed them by CO_2 inhalation when tumors reached 2-3 cm in diameter, or when the mice showed signs of illness such as weight loss, dyspnea, or lameness. Mice that did not develop tumors or become ill were observed for six months, then killed.

We calculated latency periods for tumor development following subcutaneous and intramuscular injections as the time taken for tumors to reach 1 cm in diameter. For intravenous, intracardial, and intraperitoneal injections, we calculated latency periods as the time between injection and the appearance of signs of illness or palpable tumors.

Collection and Processing of Tissues

Mice received a complete necropsy that included gross and microscopic examination of at least any tumors, cervical, lumbar, and mesenteric lymph nodes, thyroid gland, parathyroid glands, adrenal glands, salivary glands, trachea, lungs, heart, liver, spleen, pancreas, and kidneys. In some mice, portions of the musculoskeletal system and spinal cord were also examined. Specimens of all tissues were fixed in neutral buffered 10% formalin, processed by routine histologic methods, and imbedded in paraffin blocks. Six micron sections were cut and stained with hematoxylin and eosin for general microscopic examination. In particular, the lungs from mice that had received intravenous or intracardial injections were imbedded in their entirety, and multiple sections were examined for metastatic tumors.

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Table 1. Origin of Cell Lines and Strains[a]

Recipient Cell Line	Transforming ras Oncogene	Injected Cells	Reference
MSU-1.1	K-ras	K-ras-1a K-ras-2aT	11
	N-ras	N-ras-3T N-ras-5T	33
	H-ras	H-ras-2T H-ras-3T	15
	(spontaneous transformant)	L55I-3T	Milam: (personal communication)
GM637	H-ras	SV-T1/T	15
KMST-6	H-ras	K1-T1/T	15

[a]All cell lines used in these experiments were derived from subcutaneous tumors produced by their respective ras-transformed cell strain (7).

Table 2. Experimental Metastasis of ras-transformed MSU-1.1 Cells, Spontaneously-transformed MSU-1.1 cells, and Sarcoma-derived Cells[a]

Cell Strain or Cell Line Injected	Mice with Tumors/Mice Injected	Rate of Metastasis	Mean Latency Period
K-ras-1aT	4/13	31%	133 days
K-ras-2aT	1/10	10%	138 days
N-ras-3T	9/10[b]	90%	78 days
N-ras-5T	5/10	50%	115 days
H-ras-3T	0/9	---	---
H-ras-2T	4/9	44%	133 days
L55I-3T	3/11	27%	76 days
HT1080	9/10[c]	90%	85 days

a. The data shown here represent the pooled results of two separate experiments.

b. The tenth mouse, killed 45 days after injection because of weight loss, had no tumors.

c. The tenth mouse, killed 56 days after injection because of a bacterial infection, had no tumors.

Table 3. Experimental Metastasis of H-ras-transformed Human Fibroblasts[a]

Parental Cell Line	Cell Strain or Cell Line Injected	Mice with Tumors/Mice Injected	Rate of Metastasis	Mean Latency Period
MSU-1.1	H-ras-3T	0/9	---	---
	H-ras-2T	4/9	44%	133 days
KMST-6	K1-T1/T	7/9[b]	78%	65 days
GM637	SV-T1/T	0/10	---	---

a. The data shown here represent the pooled results of two separate experiments.

b. An tenth mouse that died with signs of metastatic disease 55 days after injection was unavailable for necropsy.

Table 4. Distribution of Metastatic Tumors Produced by ras-transformed Cell Strains, a Spontaneously-transformed Cell Line, and a Sarcoma-derived Cell Line

Cell Strain or Cell Line Injected	Tumor Location
K-ras-1aT	muscles of rear legs (3/4) peritoneum, kidneys, pancreas, stomach, adrenals, lumbar vertebra (1/4)
K-ras-2aT	muscles of rear legs (1/1)
N-ras-3T	muscle of rear legs (8/9) muscle of abdominal wall (3/9) dorsal thoracic cavity (not lung) (1/9)
N-ras-5T	muscle of rear legs (1/5) lungs (single tumor) (4/5)
H-ras-2T	muscle of rear legs (4/4)
K1-T1/T	muscle of rear legs, back (1/7) lungs (multiple tumors) (6/7)
L55I-3T	muscle of rear legs (3/3)
HT1080	lungs (single tumor) (6/9) muscle of rear legs (3/9)

Table 5. Distribution of Tumors Produced by Intravenous or Intracardial Inoculation of N-ras-transformed MSU-1.1 Cell Strains

Cell Strain Injected	Route of Injection	Mice with Tumors/ Mice Injected	Mean Latency Period	Tumor Location
N-ras-3	IV	1/4 [a]	200 days	lungs (multiple tumors)
	IC	2/4 [b]	148 days	endocardium (1/2) abdominal muscles, peritoneum, kidney, thoracic wall (1/2)
N-ras-3T	IV	0/5 [c]	---	---
	IC	4/6	151 days	thoracic wall (4/4) abdominal muscles, peritoneum, kidney, adrenals (1/4)
Combined results	IV	1/9	200 days	lungs (multiple tumors)
	IC	6/10	150 days	thoracic wall (5/6) abdominal muscles, peritoneum, kidney (2/6) adrenals (1/6) endocardium (1/6)

a. Two mice that died 6 days and 35 days after injection had no tumors.

b. Two mice that died 29 days and 60 days after injection had no tumors.

c. One mouse that died 4 days after injection had no tumors.

Table 6. Tumor Formation by N-ras-transformed MSU-1.1 Cell Strains Injected Intramuscularly or Subcutaneously

Cell Strain Injected	Route of Injection	No. cells Injected	Mice with Tumors/ Mice Injected	Mean Latency Period
N-ras-3	IM	10^5	4/4	49 days
	SQ	5×10^6	4/4	48 days
N-ras-3T	IM	10^5	3/5	99 days
	SQ	5×10^6	5/6 [a]	86 days

a. One mouse that died 63 days after injection had no tumor.

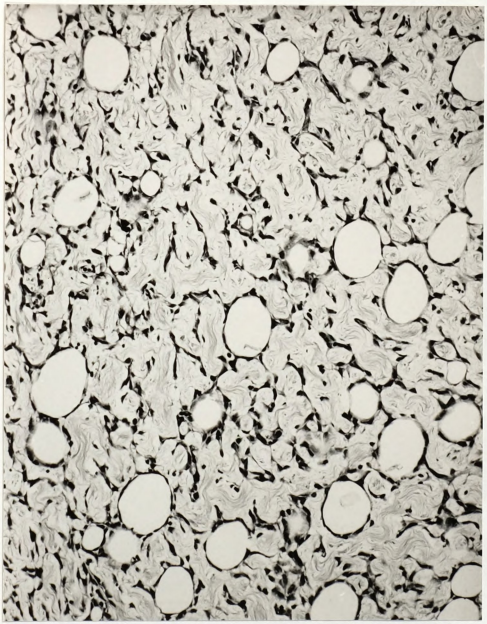


Figure 1. Myxoid sarcoma produced by K-ras-transformed MSU-1.1 cells (cell strain K-ras-1aT) injected into the peritoneal cavity. Empty circles are entrapped adipocytes (HE, 200x).

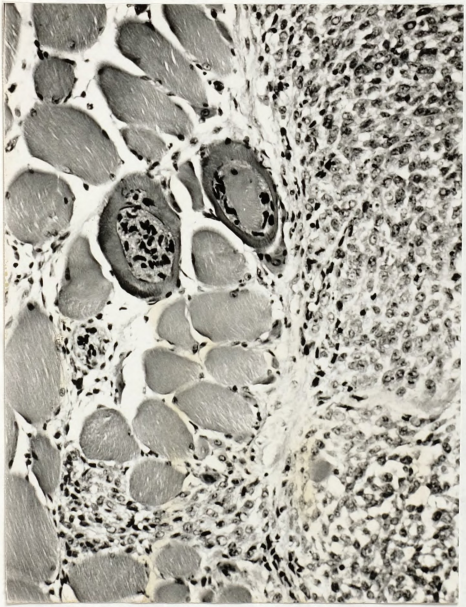


Figure 2. Spindle cell sarcoma produced by K-ras-transformed MSU-1.1 cells (cell strain K-ras-1aT) injected into the tail vein. Compare with myxoid sarcoma produced by the same cells injected into the peritoneal cavity (Figure 1).

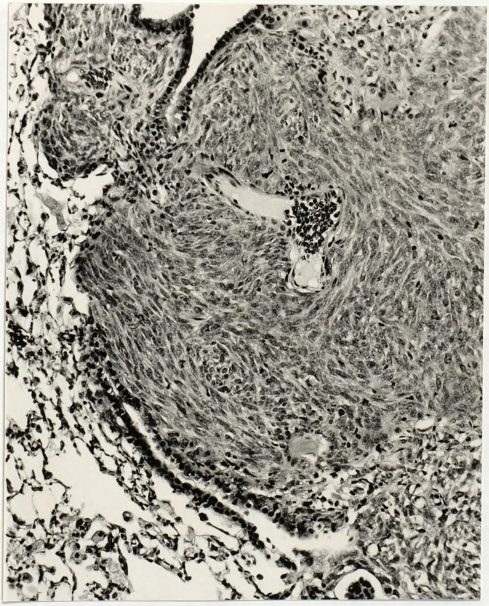


Figure 3. Spindle cell sarcoma in lungs produced by N-ras-transformed MSU-1.1 cells (cell strain N-ras-3T) injected into the tail vein. Compare with pleomorphic sarcoma produced by the same cells injected subcutaneously (Figure 7) (HE, 200x).

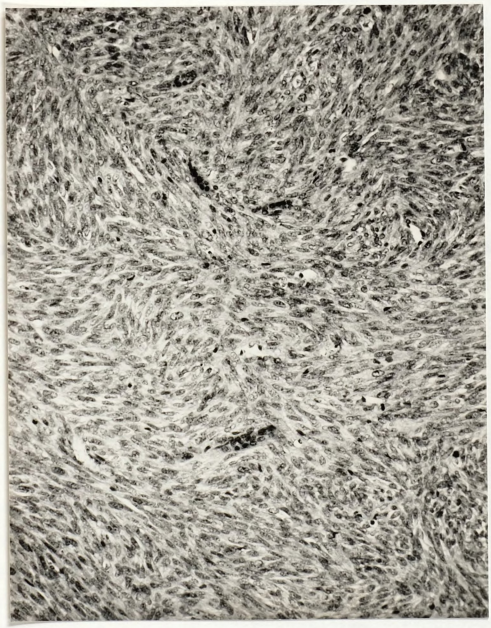


Figure 4. Spindle cell sarcoma produced by N-ras-transformed MSU-1.1 cells (cell strain N-ras-3T) injected into the peritoneal cavity. This tumor invaded the kidney; note renal tubules (HE, 200x).

APPENDIX

ATTEMPTS TO CAUSE HUMAN FIBROBLASTS TO ACQUIRE INFINITE LIFE SPAN BY TREATMENT WITH ETHYLNITROSOUREA OR 5-AZACYTIDINE

BACKGROUND AND LITERATURE REVIEW

Carcinogenesis, Transformation, and Cell Life Span

Transformation is a term borrowed from microbiology, where it refers to the transfer of genetic information between two bacteria, with acquisition of a new phenotype by the recipient. An example of bacterial transformation would be the acquisition of antibiotic resistance by a bacterium that received a plasmid bearing a drug metabolizing gene. Transformation of eukaryotic cells is more broadly defined as the acquisition of a new phenotype resulting from some change in the genome of the cell (Ponten, 1976). Cell transformation may follow the receipt of new genetic information in the form of a transfected DNA fragment, viral or proviral genome, or an entire chromosome acquired during cell fusion. Transformation also may follow the loss of DNA or alterations in DNA sequence. Even a change as small as a single base substitution may transform cells.

Obviously there are thousands of phenotypic characteristics that could be altered in transformed cells. Clues about which of them might be important in the process of carcinogenesis have come from the study of tumors and tumor-derived cell lines. For instance, many tumor-derived cells form dense, piled-up clusters of cells, called foci, when they grow on a monolayer of normal fibroblasts, while normal cells do not. This suggests that normal cells which have acquired the ability to form foci (been transformed to focus formation) as a result of some in vitro manipulation have gained a characteristic of tumor cells, and this reasoning has prompted investigators to study the molecular mechanisms

involved in focus formation and the agents which can induce that particular phenotype. For the same reason, investigators have studied transformation to other phenotypic endpoints, such as altered cell shape (morphologic transformation), reduced growth factor requirements (transformation to growth factor independence), ability to grow in soft agar (transformation to anchorage independence), increased proliferative capacity (transformation to infinite life span, or immortalization), ability to form tumors in an appropriate host (transformation to tumorigenicity), and ability to form progressively-growing malignant tumors (malignant transformation).

Several lines of evidence suggest that the acquisition of an infinite life span, commonly referred to as establishment or immortalization, is a prerequisite for full malignant transformation. Part of the evidence comes from studies of spontaneous and induced tumors; in contrast to normal cells, cells derived from tumors often have an infinite life span (Dobrynin, 1963; Ponten and Saksela, 1967; McAllister et al., 1969; Giard et al., 1973; Rheinwald and Beckett, 1981). Cancer cells presumably escape from the normal mechanisms that limit cell proliferation in various ways, and the mechanism of immortalization may well differ for different cells (Hull, 1981). Nevertheless, the observation that infinite life span is a common characteristic of many cancer cells suggests that acquisition of an infinite life span may be fundamental to the process of carcinogenesis (Smets, 1980).

Evidence of a connection between infinite life span and malignant transformation comes from several sources. Lee et al. (1989) studied two strains of mice and demonstrated a positive correlation between the frequency with which their liver cells give rise to infinite life span clones in vitro and the frequency of spontaneous liver tumor formation in vivo. Acquisition of an infinite life span seems regularly to accompany tumorigenic transformation induced in vitro by chemicals (Berwald and Sachs, 1965; Igel et al., 1975; Benedict et al.,

1975; Newbold et al., 1982), radiation (Borek, 1980), or viruses (Ponten, 1971; Shin et al., 1975; Petit et al., 1983), as well as spontaneous transformation in vitro (Ponten, 1971; Barrett, 1980; Mukherji et al., 1984; Gorman et al., 1984; Kraemer et al., 1986). In addition, acquisition of an infinite life span appears to be an absolute requirement for tumorigenic transformation induced in vitro by oncogene transfection (Rassoulzadegan et al., 1982; Land et al., 1983; Newbold and Overell, 1983; Ruley, 1983; Spandidos and Wilkie, 1984; Jenkins et al., 1984; Yoakum et al., 1985; Namba et al., 1988; Seremetis et al., 1989; Fry et al., 1989; Wilson et al., 1989; Hurlin et al., 1989).

O'Brien et al. (1986), Namba et al. (1988), and Klein (1979) all suggest that, at least with human cells, senescence is the key limiting factor in malignant transformation in vitro. Klein and Namba et al. even propose that the process of initiation in multi-stage carcinogenesis in vivo creates preneoplastic cells with an infinite life span that subsequently undergo further changes to become tumorigenic. The acquisition of phenotypic characteristics is often temporally separated in vivo into early events (initiation) and late events (promotion and progression), but such distinctions are difficult to make in vitro. For example, Land et al. (1983) have demonstrated that, although an infinite life span is a prerequisite for malignant transformation of fibroblasts, it makes no difference whether the cells acquire this characteristic "early" or "late".

On a theoretical level, multi-step models of carcinogenesis require that cells acquire an extended or infinite life span if tumors are to arise in vivo. To illustrate, consider a simple multistep model in which a normal cell must undergo two independent mutation-like events to become a malignant tumor cell. If the phenotypes associated with these events are designated A and B, then the normal cell is A^-B^- and its malignant progeny is A^+B^+. Assuming that each mutation-like event occurs spontaneously once per 10^6 cells per generation, then the transformation process can be diagrammed as in Figure 1.

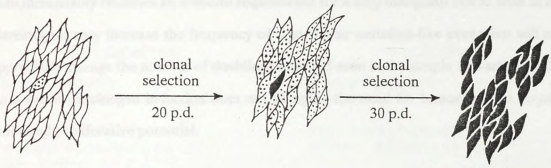


Figure 1. Model of human fibroblast transformation. In this model, malignant transformation results from 2 mutation-like events (phenotypes A and B). Note that unless the A or B phenotype extends the cells' life span beyond the normal 50 p.d., a tumor large enough to be life-threatening will not develop. If more than two mutation-like events are required, a tumor will not develop at all. (p.d. = population doublings)

To derive 10^9 malignant cells from 10^6 normal cells requires the cells to undergo 50 population doublings (pd). However, human fibroblasts have an in vitro life span of only 50-70 pd (Hayflick, 1974) which is reduced by 10-20 pd when they are clonally selected (McCormick, unpublished studies), and their life span in vivo appears similar (Cristofalo and Stanulis-Praeger, 1982). Consequently, if the original A^-B^-cells in this model are human fibroblasts then the malignantly transformed A^+B^+ cells that make up the resulting fibrosarcoma are very near the end of their life span. In other words, this 1-cm-diameter fibrosarcoma, which may be clinically undetectable and is far from life-threatening, is made up of very old cells. The tumor cannot grow much larger, let alone recur following surgical excision or grow at a metastatic site, unless the A^+ or B^+ phenotype has conferred upon the cells an infinite life span. If more than two mutation-like events are required for malignant transformation, or if those events occur less often than once per 10^6 cells per generation,

then immortality becomes an absolute requirement for a fully malignant cell to arise at all. Carcinogens may increase the frequency of a particular mutation-like event, but will not appreciably change the number of doublings required even in the simple two-stage model. As a result, carcinogen induction does not abrogate the need for tumor cells to acquire unlimited proliferative potential.

Control of Cell Life Span

Although acquisition of an infinite life span seems central to the process of carcinogenesis, we know comparatively little about the mechanisms that govern cell life span. We do know, as already mentioned, that normal vertebrate cells have a finite life span in vitro (Hayflick, 1965 and 1974) and apparently also in vivo (Cristofalo and Stanulis-Praeger, 1982). The process whereby cells accumulate deleterious changes as they age, culminating in loss of proliferative potential and/or cell death, is termed senescence (Yanishevsky and Stein, 1981; Macieira-Coelho and Azzarone, 1982; Stein et al., 1985; Cristofalo et al., 1986). The life span of cells in vitro generally falls within a narrow range that is a function of the number of population doublings the cells have undergone, rather than chronological time (Kajik, 1979). Life span is determined by the interplay of genetic and environmental factors; senescence is therefore the combined result of both genetically-determined and stochastic events, rather than being strictly a deterministic process (Macieiro-Coelho, 1980).

Cell fusion studies indicate that the phenotype of finite proliferative potential is dominant (Pereira-Smith and Smith, 1982 and 1983) but the molecular mechanisms that limit cell life span are obscure. Hypotheses usually center around the gradual accumulation of deleterious changes in DNA and proteins (error catastrophe hypotheses [Orgel, 1973; Laughrea, 1982]) or a predetermined, genetically-controlled event or series of events (genetic clock hypotheses [Hayflick, 1965]). The main features of these two theories are listed in Table 1.

Table 1. Contrast Between Proposed Mechanisms Limiting Cell Life Span

Error Catastrophe	Genetic Clock
Life span limited by "information loss"	Life span limited by a "genetic clock"
Process is stochastic, due to synthesis errors, wear-and-tear	Process is pre-programmed (planned obsolescence)
Aging = accumulation of damage	Aging = terminal differentiation
Immortality = acquired ability to avoid/repair damage	Immortality = failure to mortalize (program malfunction)

Numerous types of "errors" have been observed in aging cells, including the accumulation of extrachromosomal DNA sequences (Schmookler-Reis et al., 1983), loss of repetitive DNA sequences (Schmookler-Reis and Goldstein, 1980), loss of DNA from the margins of chromosomes (Good, 1977), decreased DNA methylation (Wilson and Jones, 1983), loss of gene repression activity (Smith and Lumpkin, 1980), and loss of flexibility of the protein synthetic pathway to respond to growth factors (Ballard and Read, 1985). The various changes in DNA associated with aging of cells were recently reviewed (Maciera-Coelho, 1984). However, protein synthesis (translation) errors do not increase as cells age (Harley et al., 1985) and the possibility exists that observed "errors" are only a secondary consequence of aging, rather than causally related to it (Thompson and Holliday, 1975).

The genetic clock hypothesis proposes a pre-programmed signal that either inactivates key genes involved in proliferation or activates genes involved in differentiation, thereby "mortalizing" the cell (Shall and Stein, 1979). In this model, aging is usually considered a differentiation process and cell death is interpreted as terminal differentiation (Bell et al., 1978; Kantermann and Bayreuther, 1979). There is evidence for the production

of an intracellular protein that inhibits proliferation (Hull, 1981; Burmer, et al., 1982; Drescher-Lincoln and Smith, 1984; Lincoln et al., 1984; Pereira-Smith et al., 1985; Stein et al., 1985; Lumpkin et al., 1986; Smith et al., 1987), as well as for inhibitory factors in serum that act intercellularly (Loo et al., 1987). There is also evidence that random activation of genes by treatment with 5-AzaC shortens cell life span (Holliday, 1986; Fairweather et al., 1987), although whether this represents an acceleration of a normal program of gene activation is unknown.

The various hypotheses concerning the control of cell life span are not mutually exclusive; rather, cell proliferation is probably regulated at multiple points in the cell cycle, which Hull (1981) called restriction points. Passage through restriction points is regulated by specific molecules that may be stimulatory or inhibitory (Yanishevsky and Stein, 1981; Pardee, 1987).

Immortalization of Nonhuman Cells

The ability to derive infinite life span cell lines in vitro differs widely, depending upon the species from which the cells are taken (Terzi and Hawkins, 1975: Macieira-Coelho et al., 1977). For example, spontaneous immortal variants regularly arise in mouse fibroblast cultures (Todaro and Green, 1963; Berwald and Sachs, 1965; Reznikoff et al., 1973) and numerous established cell lines exist such as the various 3T3 fibroblasts lines used in in vitro assays for transforming genes. In contrast, spontaneous infinite life span variants arise less commonly in cultures of rat and Syrian hamster fibroblasts, and almost never appear in chicken fibroblast cultures (Ponten, 1971). Despite extensive studies with human fibroblasts, spontaneously-arising infinite life span variants have been very rarely reported (Thielmann et al., 1983; Mukherji et al., 1984), as discussed by DiPaolo (1983).

Infinite life span non-human fibroblast lines have been generated by treatment with carcinogenic chemicals (Oshimura et al., 1986), infection with viruses, and transfection with oncogenes derived from viruses or tumors (Houweling et al., 1980; Rassoulzadegan et al., 1982; Jenkins et al., 1984; Spandidos and Wilkie, 1984; Griffin and Karran, 1984; Connan et al., 1985; Shimada et al., 1987; Rovinski and Benchimol, 1988). Most of the time, the infinite life span lines exhibit other characteristics of transformed cells, such as anchorage independence, focus formation, altered morphology, or tumorigenicity in immunodeficient or histocompatible hosts, but there are exceptions. Rat kidney cells transfected with a fragment of human adenovirus type 5 DNA by Houweling et al. (1980), rat embryo cells transfected with the gene for the polyoma virus large T antigen by Rassoulzadegan et al. (1982), and rat chondrocytes transfected with the mouse p53 gene by Jenkins et al. (1984) all acquired an infinite life span without showing changes in morphology or growth characteristics. Also, Newbold et al. (1982) immortalized hamster dermal fibroblasts by treating them with chemical carcinogens and the infinite life span cells did not exhibit other characteristics of transformed cells. In most of these examples the infinite life span cells gradually became "fully transformed" with passage in vitro. In contrast, when Griffin and Karran (1984) immortalized monkey kidney epithelial cells by transfection with a fragment of the Epstein-Barr virus genome, the cells remained stable in culture and did not progressively acquire other characteristics of transformed cells, perhaps reflecting greater genetic stability of primate cells compared to rodent cells.

Immortalization of Human Cells

Unlike rodent cells, human cells seem resistant to establishment in vitro as infinite life span cell lines. The sole exception seems to be human B-lymphocytes, which are readily immortalized by infection with Epstein-Barr virus (Spear, 1983). EBV-immortalized

lymphocytes do not show morphologic or karyotypic changes and are nontumorigenic in nude mice (Nilsson and Ponten, 1975; Nilsson et al., 1977). The mechanism of immortalization by EBV is unknown.

Other viruses can immortalize human cells, but the connection between infection and the acquisition of an infinite life span is less direct. Human embryonic kidney cells can be immortalized by infection with human papovavirus, but with a very low frequency (Purchio and Fareed, 1979). Human keratinocytes (Rhim et al., 1985) or fibroblasts (Sack, 1981) infected with SV40 virus, exhibit morphologic changes and altered growth characteristics in vitro but rarely give rise to infinte life span clones. Cultures of SV40 virus-transformed fibroblasts have a slightly prolonged but finite life span; rarely (< 1 in 10^7), an infinite life span variant will emerge at senescence (Huschtscha and Holliday, 1983). Similarly, proliferating clones of cells appear rarely in senescent cultures of SV40 virus-infected keratinocytes. Infection with SV40 virus is thought to prevent cells from entering a normal viable senescent state (Stein, 1985). How this change predisposes cells to immortalization is unknown, but clearly immortalization is not a direct effect of SV40 virus infection.

The life span of human cells in culture can be prolonged by hydrocortisone (Grove and Cristofalo, 1976), but only very few investigators have reported the immortalization of human cells in culture by treatment with chemical carcinogens or radiation (Igel et al., 1975; Kakunaga, 1978; Namba et al., 1978 and 1981; Stampfer and Bartley, 1985; Namba, 1985). In only two instances were the parental cells preserved and the life span assay described. Namba et al. (1978 and 1981) reportedly generated three infinite life span fibroblast lines by prolonged treatment of human embryo fibroblasts with 4-NQO and gamma radiation. McCormick and Maher (1988) compared these cell lines to the parent cells by a variety of methods and concluded that the two of the lines are identical to each other, but that the two distinct lines obtained by these investigators are derived from the parent cells and represent

a bona fide example of immortalization. These infinite life span human cells exhibit other characteristics of transformed cells, such as morphologic changes, altered growth characteristics, and karyotypic abnormalities, but they are nontumorigenic in nude mice. Kakunaga (1978) reported isolating 15 infinite life span human fibroblast lines from foci of morphologically-altered cells appearing in cultures of dermal fibroblasts treated with 4-NQO and MNNG. The majority of these cell lines were later lost, but four were available for study (Leavitt and Kakunaga, 1980; Varma and Leavitt, 1988). However, these cells were later found not to be derived from the putative parent cells (McCormick and Maher, 1988). Instead, they are identical to the fibrosarcoma-derived cell line 8387, which itself already has an infinite life span (McCormick et al., 1988).

McCormick and his colleagues generated an infinite life span fibroblast line, MSU-1.1 (McCormick et al., 1989). This cell line arose within a clonal population of normal human foreskin fibroblasts transfected with a plasmid containing the neo gene and a v-myc oncogene. Several G418-resistant cells were isolated and shown to express the v-myc oncogene. The majority of the cultures went through crisis and a single cell line emerged. The cells grew slowly during the first several months in culture, but subsequently resumed a normal growth rate. These have been designated MSU-1.1 fibroblasts. MSU-1.1 fibroblasts have the same isozyme and HLA antigen pattern as the parent cells, are morphologically similar, and are nontumorigenic in nude mice. Their karyotype has been stable for over 200 population doublings. They have a near-diploid DNA content, but differ from the parent cells in having two marker chromosomes created by rearrangements between chromosomes 1 and 11, and 12 and 15. The relationship between the transfected myc oncogene and the cells' infinite life span is under investigation. Since a diploid infinite life span cell line (MSU-1.0) was derived from the same clone of cells transfected with the v-myc oncogene (McCormick, unpublished studies), the chromosomal rearrangements observed in the MSU-1.1 cell line are not causally involved in the infinte life span phenotype.

INTRODUCTION

Much evidence suggests that immortalization, or the acquisition of an infinite life span by cells, is fundamental to the process of carcinogenesis (for discussion, see Klein, 1979; Smets, 1980; O'Brien et al., 1986; Namba et al., 1988; McCormick and Maher, 1988). Indeed, the difficulty of transforming human cells to tumorigenic cells in vitro compared to mouse or rat cells appears directly related to the relative resistance of human cells to spontaneous or induced immortalization (DiPaolo, 1983). In contrast to mouse fibroblasts, infinite life span variants rarely appear in cultures of human fibroblasts and apparently are difficult to induce by treating cells with chemical carcinogens or radiation. On the other hand, available evidence on cell proliferation suggests that passage of human fibroblasts through the cell cycle is regulated at only a few key steps, called restriction points (Hull, 1981), and that passage through these restriction points is mediated by the products of a few key genes (Pardee, 1987; Pereira-Smith and Smith, 1988). Theoretically, mutation-like events that alter such growth regulating genes might induce or allow unregulated cell proliferation by abolishing the molecular controls that normally limit it.

I hypothesized that human fibroblasts could be immortalized by a mutation-like event in a growth regulating gene; that is, by a rare, permanent, heritable change in gene expression resulting from an alteration in the regulatory or coding sequences of the gene. In other words, I postulated that human fibroblasts could acquire an infinite life span (lose the ability to senesce or undergo terminal differentiation) in the same way they could gain or lose any other phenotypic characteristic. This hypothesis led me to two others: first, that infinite life span variants arise spontaneously in human fibroblast cultures at some low but detectable frequency, and second, that the frequency of immortalization could be increased by treating fibroblasts with agents that cause mutations or mutation-like changes in DNA. I expected

that testing these hypotheses would be no more difficult than conducting any other type of mutagenesis assay, provided two conditions could be met: infinite life span variants had to express that phenotype, and I had to be able to recognize them within fibroblast cultures.

What I needed was an assay for infinite life span fibroblasts. At first glance, designing an assay for infinite life span cells seemed straightforward --if cells were continually passaged in culture, infinite life span variants should emerge as proliferating clones at senescence, when the rest of the cells in culture ceased proliferating. But the historical evidence from a quarter century's experience with cultured human fibroblasts suggested that this approach would not work. Infinite life span variants have appeared very rarely, even among human fibroblasts treated with chemical mutagens and radiation. Investigators have taken this as evidence that human fibroblasts cannot be immortalized by mutation-like events. But I considered that previous results might indicate instead that infinite life span human fibroblasts could not be recognized under normal cell culture conditions.

If infinite life span variants do occur, then why might they not appear as proliferating clones in senescent fibroblast cultures? One possibility is that infinite life span variants are inadvertently discarded during serial passages of cell cultures. Normal cell culture procedures involve discarding or freezing back a portion of the culture at intervals. This process might be repeated 10 times over the life span of a normal fibroblast culture that undergoes about 50 population doublings. The end result of such cell culture procedures is that the majority of the cells are discarded and relatively few are carried through their entire life span. In this situation, rare variants readily can be lost. If a variant has a slight competitive disadvantage, such as a slower growth rate, it is even more likely to be lost.

Namba et al. (1988) suggested a second reason why infinite life span variants might not grow out of senescing fibroblast cultures. If expression of the infinite life span phenotype is inhibited by surrounding normal fibroblasts, such variants will remain latent and

undetected. There is evidence that this occurs. For instance, rodent fibroblasts can be immortalized by transfection with a myc oncogene (Mougneau et al., 1984). Bignami et al. (1988) found that proliferation of myc-transformed rodent fibroblasts is inhibited by contact with normal fibroblasts but not by conditioned medium from normal fibroblast cultures. Dotto et al. (1988) found that normal fibroblasts also can inhibit the in vivo proliferation of mouse keratinocytes that are malignantly-transformed by Harvey murine sarcoma virus. Studies in our own laboratory with several infinite life span human fibroblast cell lines indicate that their proliferation can be inhibited by a soluble factor in the medium of normal fibroblast cultures and possibly by direct contact with normal fibroblasts as well (Sato, personal communication).

Clearly, the problems of cell loss during passage and inhibition by normal fibroblasts in co-cultures could be eliminated if latent infinite life span cells could be identified as they appeared, removed from bulk cultures, and grown as clonal populations. Infinite life span variants then might be free to express their unlimited proliferative potential. Studies in McCormick's laboratory with the infinite life span human fibroblast line MSU-1.1 suggested a possible approach to identifying latent immortal fibroblasts, based on the calcium concentration of the culture medium. Calcium acts like a growth factor for human fibroblasts in culture. McCormick and his colleagues found that MSU-1.1 fibroblasts proliferated in low calcium medium but the parent normal fibroblasts did not (J. McCormick, personal communication). I reasoned that if the combination of infinite life span and reduced requirement for calcium was not coincidental, then ability to grow in medium with a reduced calcium content might serve as a phenotypic marker for infinite life span cells. I decided, therefore, to screen human fibroblast cultures for cells capable of proliferating in low calcium medium, clone any calcium-independent variants I found, and determine if those calcium-independent variants had infinite life spans.

Because I expected calcium-independent variants to be rare (Kraemer et al., 1986), I wanted to screen large numbers of cells. I also wanted to screen cells under conditions that prevented cell-to-cell contact, in case such contact inhibited proliferation. Screening cells on plastic dishes at clonal density would prevent cell contact but would require hundreds of dishes per experiment. Instead, I decided to grow cells suspended in soft agar so that I could screen many cells with relatively few dishes. We had found previously in the laboratory that we could achieve high cloning efficiency in soft agar if we grew normal human fibroblasts in relatively defined medium containing adequate growth factors supplied by serum or as purified supplements (Ryan et al., 1987). For my purposes, growing cells in soft agar had two additional advantages over growing them on plastic besides enabling me to screen many cells per dish. First, I could pick proliferating clones cleanly from the agar, free of contamination by normal fibroblasts. Second, I could count proliferating clones using a computerized cell counter.

In the studies reported here, I treated human fibroblasts with ENU or 5-AzaC. ENU is an alkylating agent which causes base substitutions and occasional small deletions that inactivate genes (Aust et al., 1984; Drinkwater and Kleindinst, 1986; Skopek, 1986; DuBridge et al., 1987). 5-AzaC interferes with methylation of newly-synthesized DNA; decreased methylation is generally thought to activate genes (Jones et al., 1982; Bird, 1984). I passaged both treated and untreated cells as bulk cultures to see if infinite life span variants emerged when the cultures senesced. In addition, I assayed cells at intervals by growing them in soft agar under low calcium conditions, to identify and quantify calcium-independent variants. Clones of fibroblasts that proliferated in low calcium conditions were isolated and tested to see if the calcium-independent phenotype was stable and if the cells had infinite life spans. I found that normal fibroblast cultures did indeed contain a few cells that were calcium-independent and that I could induce such variants with ENU, but that the calcium-independent phenotype was not stable and that such cells had normal life spans.

MATERIALS AND METHODS

Cells and Cell Culture

SL88 and LG1 are normal human fibroblast lines derived from neonatal foreskins. MSU-1.1 fibroblasts are an infinite life span human fibroblast line derived from a population of LG1 fibroblasts transfected with a v-myc oncogene (McCormick et al., 1989). HT1080 cells are derived from a human fibrosarcoma (Rasheed et al., 1974).

Cells were grown in 75 mm tissue culture flasks at 37°C in a 5% CO_2 atmosphere and fed with FM medium supplemented with 5-10% FCS, 100 units/ml penicillin, 100 ug/ml streptomycin, and 10 ug/ml hydrocortisone. Unless otherwise noted, cultures were fed weekly and passaged when nearly confluent. Cultures were passaged by loosening cells with trypsin, harvesting them in FM medium, counting a 0.5 ml aliquot in an automated cell counter (Coulter Electronics, Inc., Hialeah, FL) to keep track of the age of the cells in population doublings, and replating cells at 500,000 cells/flask. At each passage, cells not put back into culture were stored at -120°C.

Treatment with ENU and 5-AzaC

Cells were plated at 800,000/150 mm-diameter petri dish. ENU solution was prepared by dissolving ENU in DMSO to a final concentration of 400 mM. Cells were exposed to serum-free FM medium containing 100 uls of ENU solution (final concentration = 2 mM) for 1 hour. Control cell cultures were exposed to 100 uls of DMSO in serum-free FM medium for 1 hour. Following treatment, cells were harvested, counted, and replated into 75 mm tissue culture flasks to allow an expression period. Cells were also plated into 100 mm-diameter petri dishes to determine % survival.

For azacytidine treatment, 5-AzaC was added to cells in regular cell culture medium to a final concentration of 10 uM.

Assay for Calcium-Independent Variants

I assayed cells in 100 mm petri dishes as follows. Low calcium agar solutions were prepared by dissolving Seakem agar in McM medium prepared as described by Ryan et al. (1987), except that the calcium concentration was 0.1 mM instead of 1.0 mM. Medium contained 0.5% FCS, 1.0 mg/ml calcium-free BSA, 100 units/ml penicillin, 100 ug/ml streptomycin, 900 ng/ml dithiothreitol, 180 ng/ml glutathione, 1.9 ug/ml phosphoenolpyruvate, 8.1 ng/ml prostaglandin E1, 1 ug/ml insulin, 1.4 ug/ml $FeSO_4$, 13.5 ng/ml cholesterol, 11 ng/ml soybean lecithin, 4.5 ng/ml sphingomyelin, 4.7 ng/ml vitamin E, and 10 ug/ml hydrocortisone. I first poured a bottom layer of 2% agar and allowed it to harden. On top of this I poured a top layer of 0.3% agar containing 150,000 cells per dish. Cells were fed 24 hours after plating and weekly thereafter with the same medium used to prepare the original agar. Cells were also plated into plastic petri dishes and into agar made with 4% serum to compare cloning efficiencies. Cells on plastic and in agar made with 4% serum were fed weekly with medium containing 10% and 4% serum, respectively.

Agar dishes were examined twice weekly under a microscope for evidence of proliferation. Proliferating clones greater than 40 u in diameter were counted using a computerized agar counting system. Once counted, proliferating clones were removed in an agar plug, teased gently free of the agar, and inoculated into 60 mm culture dishes.

RESULTS

I initially tested the low calcium agar assay to see if it discriminated among cells with different calcium requirements by comparing the growth of SL88 fibroblasts, MSU-1.1 fibroblasts, and HT1080 cells. The ability of these cells to form proliferating colonies in low calcium agar (Table 2) paralleled their growth curves on plastic under low calcium conditions.

Table 2. Growth of Untreated Fibroblasts in Low Calcium Agar

	Cloning Efficiency (clones per 10^6 cells)	
Cell Line	**Plastic (10% serum)**	**Low Cal Agar (0.5% serum)**
SL88[a]	190,000	564
MSU-1.1	680,000	7,289
HT1080	290,000	43,195
SL88-2[b]	< 1,000	< 10

a. SL88 fibroblasts at age 18 population doublings

b. SL88-2 fibroblasts at age 40 population doublings

HT1080 cells grew readily in medium with reduced calcium concentration, MSU-1.1 fibroblasts grew less well, and normal fibroblasts grew very poorly. However, there were a few variants within young SL88 fibroblasts (about 6/10,000 cells) that could proliferate in low calcium conditions. I picked these clones and attempted to expand them into populations large enough to retest in low calcium agar and assay for infinite life

span. Only 1 of 21 clones, SL88-2, yielded enough cells to retest in low calcium agar; the rest senesced within 20 population doublings of being isolated as clones in agar. When SL88-2 cells were plated into agar once more, no proliferating clones were detected (Table 2), indicating that, at least in this one case, calcium-independence was not a stable phenotype.

I next treated SL88 and LG1 fibroblasts with ENU or 5-AzaC at population doublings 18 and 30, respectively. ENU treatment resulted in 18% survival of SL88 fibroblasts and 10% survival of LG1 fibroblasts. 5-AzaC was nontoxic to cells. Treated and control fibroblasts were passaged to senescence as bulk cultures. At intervals, cells were harvested and tested for growth on plastic and in agar under normal and low-calcium conditions (Tables 3 and 4). Calcium-independent variants were not detected in control LG1 cultures, nor were they induced by ENU or 5-AzaC. Calcium-independent variants did appear with increased frequency in ENU-treated SL88 cultures at population doubling 28, but 3 population doublings later these variants were no longer detectable, suggesting that the altered phenotype had been transient.

I picked proliferating clones from low calcium agar and attempted to expand them into populations large enough to retest in low calcium conditions. Nine LG1 clones failed to proliferate more than 1 or 2 population doublings. Of 12 SL88 clones, only 1 proliferated more than a few population doublings and it failed to yield enough cells to retest before senescing. None of the calcium-independent clones had an extended life span.

Table 3. Growth of ENU- and 5-AzaC-treated LG1 Fibroblasts in Low Calcium Agar

		Cloning Efficiency (clones per 10^6 cells)		
Treatment	Age[a]	Plastic (10% serum)	Agar (4% serum)	Low Cal Agar (0.5% serum)
Untreated	33	3,750	399	< 10
ENU	35	< 1,000	263	< 10
5-AzaC	33	20,000	722	< 10

a. Age is expressed in population doublings

Table 4. Growth of ENU- and 5-AzaC-treated SL88 Fibroblasts in Low Calcium Agar

		Cloning Efficiency (clones per 10^6 cells)		
Treatment	Age[a]	Plastic (10% serum)	Agar (4% serum)	Low Cal Agar (0.5% serum)
Untreated	26	60,625	2,933	< 10
	28	14,000	13,047	< 10
	30	37,500	3,331	< 10
ENU	28	60,625	5,298	125
	31	45,000	36,179	< 10
5-AzaC	26	20,000	2,429	< 10
	29	10,000	5,083	< 10

a. Age is expressed in population doublings

I also picked proliferating clones from regular agar fed with 4% serum to see if they would prove immortal. Of 9 LG1 clones and 63 SL88 clones, none had an extended life span; only 3 SL88 clones underwent more than a few population doublings, and these ultimately senesced.

No infinite life span variants emerged from any of the cloned populations at senescence. When the bulk cultures of treated and control fibroblasts senesced at about population doubling 40, I refed them weekly and maintained the cultures for several months. During that time, foci of proliferating cells occasionally appeared. I picked these foci, transferred them to 60 mm culture dishes, and fed them weekly with medium containing 10% serum. The 38 such foci I isolated underwent 5-15 more population doublings but all ultimately senesced.

DISCUSSION

The agar assay designed for these studies did discriminate among fibroblasts on the basis of calcium requirements. Unlike normal fibroblasts, infinite life span MSU-1.1 fibroblasts proliferated in 0.1 mM calcium, but not as efficiently as fibrosarcoma-derived cells. Young cultures of normal fibroblasts contained a few cells with reduced calcium requirements, but these variants disappeared or were lost as the culture aged and was passaged. Treatment with the mutagen ENU could enhance the number of calcium-independent variants, but only transiently. These observations do not support the idea that reduced requirement for calcium results from the activation or inactivation of a specific gene. Instead, they suggest that the calcium-independent variants identified in this assay represent one end of a spectrum of biological variation.

My failure to derive infinite life span fibroblast cell lines from chemically-treated cultures is similar to the experience of others. Hypomethylating agents reportedly **reduce** rather than prolong the life span of human fibroblasts in bulk culture (Holliday, 1986; Fairweather et al., 1987), although no attempt was made in previous studies to look for rare infinite life span variants. With mutagens, infinite life span cells are produced only by prolonged or repeated treatment, and they typically have other transformed characteristics such as altered morphology and gross karyotypic abnormalities (Milo and DiPaolo, 1978; Borek, 1980; Namba et al., 1978 and 1981; Namba, 1985: Stampfer and Bartley, 1985). This may mean that human cells acquire an infinite life span infrequently because multiple or gross genetic changes are required (Cairns, 1981). But it may mean instead that human cells only **appear** to immortalize infrequently because infinite life span variants cannot be recognized under normal cell culture conditions unless they also acquire additional characteristics like altered morphology, or unless most of the normal

cells in culture with them are destroyed (DiPaolo, 1983). If that is true, then immortalization may require only a single genetic change, not many. Human fibroblasts exposed to a single treatment with propane sultone, MNNG, or 4-NQO can be transformed to growth factor independence and anchorage independence (Silinskas et al., 1981; Miyaki et al., 1982), suggesting that altered growth characteristics could result from a single mutational event. Stampfer and Bartley (1985) regularly extended the life span of human mammary epithelial cells be 2 treatments with BPDE, and even generated two infinite life span cell lines that way.

In these experiments I tried to find potentially-immortal cells and remove them from co-culture with normal fibroblasts. My inability to do so does not mean they did not exist, but only that I did not hit upon the right way to look for them. Possibly I failed because there is no connection between reduced calcium requirement and infinite life span. Admittedly, my results suggest that such a connection does not exist, since all the calcium-independent variants I isolated senesced. But in no case was the calcium-independent phenotype stable. These studies therefore do not rule out that a stable, permanent reduction in calcium requirement is related to infinite life span. On the contrary, McCormick and his colleagues have found that MSU-1.1 fibroblasts share the stable phenotypes of infinite life span and calcium-independence, and Mougneau et al. (1984) found that myc-transfected rat fibroblasts acquire both reduced growth factor requirements and an infinite life span. The polyoma virus large T antigen produces the same effect as myc when introduced into rat fibroblasts (Rassoulzadegan et al., 1982). When Griffin and Karran (1984) transfected monkey kidney cells with a fragment of Epstein-Barr virus, the cells became able to grow in very low serum and acquired an infinite life span. Immortalization of human fibroblasts by SV40 virus also frequently reduces their requirement for growth factors (Sack, 1981). Thus, although

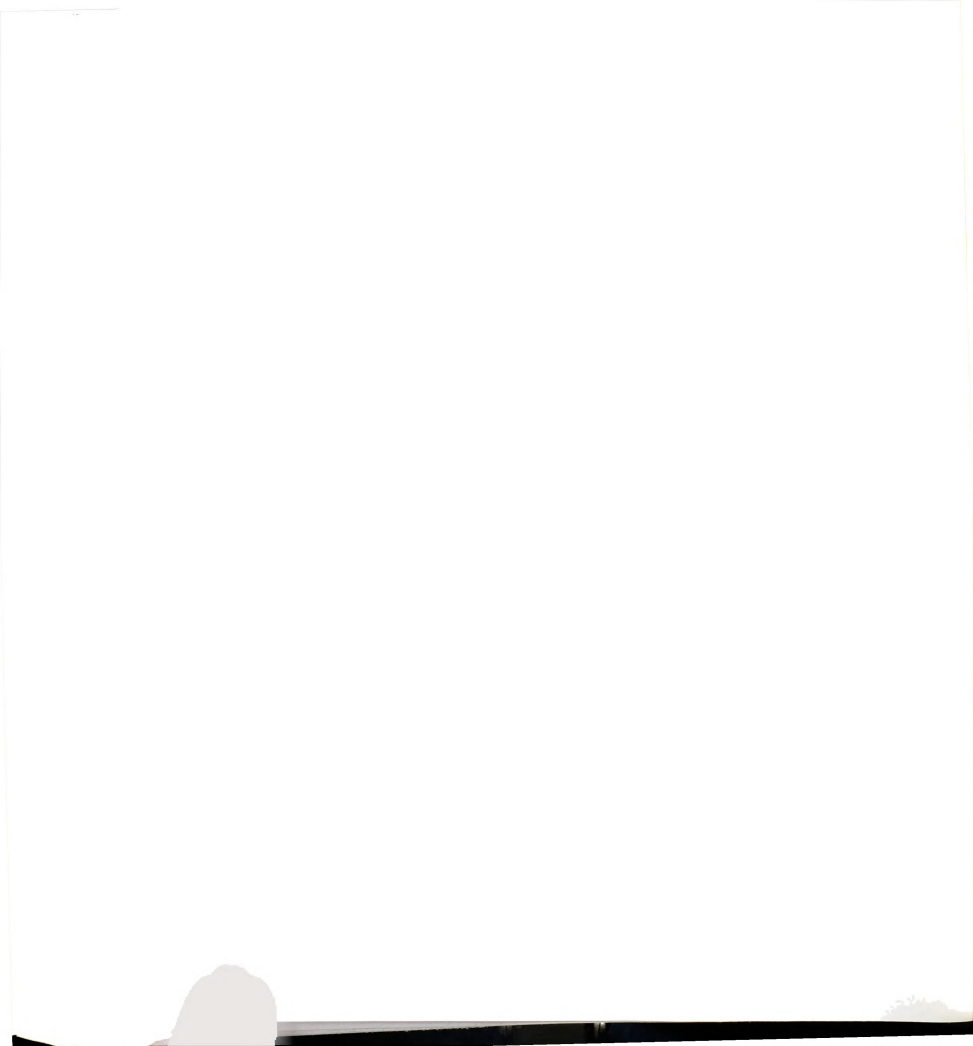
immortalization is not always linked to reduction in growth factor requirements (Newbold and Overell, 1983), the connection between the two warrants further exploration.

My inability to find infinite life span fibroblasts also may mean that expression of their infinite life span was inhibited by mortal fibroblasts in the same culture, even though cell-to-cell contact was prevented by growing cells suspended in soft agar. Experiments are currently underway to investigate this possibility and to find other ways to search for infinite life span variants. Several possibilities suggest themselves, such as using phorbol esters to block gap junction-mediated cell-to-cell communication. Blocking inhibition caused by a soluble factor released from normal fibroblasts would be more difficult, but if the factor could be partially purified then antibodies might be generated that could be used to neutralize it.

SUMMARY

In these studies I attempted to address the question of whether human fibroblast cultures contain spontaneously-arising infinite life span variants that go undetected by current cell culture procedures, and whether such variants could be induced by activating or inactivating genes with mutagens or hyopmethylating agents. I attempted to find infinite life span variants by looking for cells able to grow in low calcium conditions. By growing cells in soft agar to prevent cell-to-cell contact I was able to isolate calcium-independent variants, but they were not phenotypically stable and did not have extended life spans. Unfortunately, by the nature of my original question, negative results did not indicate that infinite life span variants were absent from fibroblast cultures, but only that I was unable to identify them.

I undertook the search for infinte life span variants with some hope for success, partly because an infinite life span fibroblast line had arisen in McCormick's laboratory from the same LG1 fibroblasts that I used in my experiments. I found myself in much the same position as Professor J.L.B. Smith in 1938. In that year he identified a fish pulled from the waters off Madagascar as a coelocanth, a prehistoric species thought extinct for 60 million years. Despite intense efforts he did not find another one until 1952. Since then, dozens of specimens of this prehistoric fish have been captured and studied (Fricke, 1988). I suspect the search for infinite life span fibroblasts will ultimately meet with the same success. All evidence points to infinite life span as a prerequisite for malignant transformation (McCormick and Maher, 1988). If that is true, then human cells must acquire an infinite life span as readily as rodent cells in vivo, since tumors arise frequently in both humans and rodents. That human cells don't appear to do so as readily as rodent



cells in vitro probably has more to do with cell culture techniques than with intrinsic species differences.

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