Analysis of p53 Gene Mutations in Keloids Using Polymerase Chain Reaction–Based Single-Strand Conformational Polymorphism and DNA Sequencing

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Background: Keloids are the result of a dysregulated wound healing process. They are characterized by the formation of excess scar tissue that proliferates beyond the boundaries of the original wound. Somatic mutations of p53 have been implicated as causal events in up to 50% of all human malignancies. In addition, p53 has been shown to play an important role in controlling cell proliferation and apoptosis. We hypothesize that mutations in p53 can lead to a hyperproliferative state that can result in keloid formation.

Objective: To detect p53 DNA mutations in tissues and cultured fibroblasts from skin lesions of 7 patients with keloids.

Design: The polymerase chain reaction followed by single-strand conformational polymorphism analysis and direct DNA sequencing were used to detect p53 gene mutations.

Setting: The Department of Dermatology, Henry Ford Hospital, Detroit, Mich.

Patients: Seven patients with keloids seen for routine surgical excision of their lesions. Normal DNA specimens were obtained from buccal smears and healthy skin samples from these patients.

Results: Mutations in the p53 gene were identified in all patients by polymerase chain reaction followed by single-strand conformational polymorphism analysis and subsequently confirmed by DNA sequencing. A mutation in exon 5 resulting in amino acid substitution was found in 1 of the patients in keloid tissue and cultured keloid fibroblasts (codon 156, CGC→CCC, arginine→proline). Frameshift mutations in exons 5 and 6 caused by the insertion or deletion of a nucleotide at different positions were found in 6 patients with keloids in both keloid tissues and cultured fibroblasts. Mutations in exon 4 resulting in amino acid substitution were found in all patients in both keloid tissues and cultured fibroblasts (all in codon 72, CGC→CCC, arginine→proline). No p53 mutations were detected in buccal smears or cultured fibroblasts from healthy skin samples of any of the patients.

Conclusions: Focal mutations in p53 may increase cell proliferation and decrease cell death in the dysregulated growth patterns that have been clinically documented. An understanding of the pattern of all growth dysregulation related to keloids may lead to new therapeutic strategies.

Arch Dermatol. 1998;134:963-967

The formation of keloid scars after skin trauma is a significant clinical problem particularly in the black population, in which the incidence of keloids has been estimated at 4% to 16%. Keloids are locally aggressive, and in contrast to hypertrophic scars, they invade healthy tissue. They often recur after multimodal therapy and can actively persist for many years. Keloids begin to develop following events involved in the repair of healthy tissue. Somehow, this normal repair sequence becomes dysregulated, and the evolving scar remains in the proliferative phase of healing. Keloid fibroblasts demonstrate both the reduced requirements of growth factors in vitro and the hyporesponsiveness to mediators that are inhibitory to normal fibroblasts. Despite these abnormal growth characteristics, the mechanisms of keloidogenesis are unknown.

The p53 gene on chromosome 17p13.1 encodes a 53-kd phosphoprotein that acts as a transcription factor and has tumor-suppressor functions. The wild-type gene product also has the ability to arrest growth and/or apoptosis in response to DNA injury, preventing the replication of genomes that have suffered DNA damage. Interest in p53 arises from the realization that a wide variety of sporadic human tumors associated with different genetic alterations are characterized by the aberrant expression of p53. Mutant p53 alleles appear to increase the risk of cancer.
PATIENTS AND METHODS

PATIENTS

Fresh keloid tissues were obtained from 7 patients for culture at the time of surgical excision. Normal DNA specimens from these patients were obtained from buccal smears (n = 5) and healthy skin (n = 2). All procedures were performed under the approval of the Henry Ford Health System Human Rights Committee. The medical and family histories of all patients were reviewed. Only 1 patient had a family history of cancer. This patient’s father died of colon cancer and his mother died of leukemia. None of the patients had a personal history of malignancy.

KELOID FIBROBLAST PRIMARY CULTURES

Primary cultures of healthy adult skin fibroblasts and keloid fibroblasts were generated as described by Hou et al.23 Fresh keloid tissue specimens were kept in cold Dulbecco’s modified Eagle medium (with 10% fetal bovine serum and 2% penicillin and streptomycin) and transferred to our laboratory within 2 hours of excision. The tissue was cut into small pieces in a sterile culture dish and transferred to a 25-mL flask with 3 mL of dispase (GIBCO BRL, Life Technologies Inc, Gaithersburg, Md) solution (2.4 U/mL in DMEM media). The flask were incubated overnight at 37°C in a shaker incubator. Samples were then centrifuged for 5 minutes at 1400g, transferred into a fresh flask with DMEM medium, and put in a 37°C incubator (95% air and 5% carbon dioxide). The primary culture was checked every 4 days and the medium was changed when necessary. When the primary cell lines were confluent, they were passed by detaching cells from the culture plate using trypsin/EDTA solution and grown in media until enough cells were produced. Studies were conducted using the passage of 3 to 6 cells to maintain comparability. The in vitro p53 positive-control squamous cell carcinoma cell line (1555-CRL, American Tissue Culture Collection, Rockville, Md) was maintained in culture as recommended by the supplier.

KELOID FIBROBLAST PRIMARY CULTURES

DNA ISOLATION

Genomic DNA was isolated from human keloid tissues, keloid fibroblasts, and normal fibroblasts in culture using the established protocol for the DNAzol reagent (GIBCO BRL, Life Technologies Inc).

SAMPLE AMPLIFICATION AND SSCP ANALYSIS

A 10-mL aliquot of the solution containing genomic DNA was subjected to PCR followed by SSCP in a total volume of 50 mL of reaction mixture containing 10-mmol/L Tris-hydrochloride (pH 8.3), 50-mmol/L potassium chloride, 1.5-mmol/L magnesium chloride, 0.01% gelatin, 1.0 µmol/L (50 pmol) of each p53 gene-specific (exons 2-11) primer (Clontech Labs Inc, Palo Alto, Calif), 200-µmol/L deoxyadenosine triphosphate, 0.5-µL α-phosphorus P 32-deoxyadenosine triphosphate, and 1.25 units of Taq DNA polymerase. Polymerase chain reactions were carried out in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn) for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. After the last cycle of amplification, the extension was continued for an additional 7 minutes at 72°C. Two microliters of the PCR products was added to 9 µL of gel-loading buffer (95% formamide, 10-mmol/L sodium hydroxide, and 0.05% xylene cyanol FF), heated at 94°C for 2 minutes, and chilled on ice. Then electrophoresis was performed on 5 µL of this sample through 0.5 times mutation detection gel solution (MDE, AT Biochem, Malvern, Pa) at 6 W for 14 hours. The DNA bands were then visualized by silver staining following the standard protocol provided by the manufacturer (Bio-Rad, Hercules, Ca).

DNA CLONING AND SEQUENCING

Amplified products were directionally subcloned into a linearized pGEM-T plasmid containing the T3 and T7 RNA polymerase promoter (as described by Clontech PCR-direct cloning system (Clontech Labs Inc). DNA was sequenced in a core facility at Wayne State University, Detroit, Mich, using the thermo sequence fluorescent primer cycle sequencing kit (Promega, Madison, Wis).24

The direct causal relationship between impaired p53 expression and tumorigenesis has recently been formally proven by generating knock-out mice with homozygous deletions in the gene. Such mice develop normally but are highly susceptible to the development of different forms of cancer.13 The discovery of germ line p53 mutations in human patients susceptible to Li-Fraumeni syndrome indicates that the loss of normal expression in this gene also plays a pivotal role in the development of tumors.14-16 A number of oncogenic viral proteins and the cellular protein mdm-2 can also form complexes with wild-type p53, initiating gene inactivation by mechanisms other than mutational loss of function.17-22 In this study, we used polymerase chain reaction/single-strand conformational polymorphism (PCR/SSCP) and DNA sequence analysis techniques to characterize p53 mutations in a series of human keloid tissues and cultured keloid fibroblasts. Focal p53 mutations in dermal fibroblasts are thought to contribute to the formation of keloids.

RESULTS

Polymerase chain reaction/single-strand conformational polymorphism analysis of exons 2 through 11 of the p53 gene was performed as described in the “Patients and Methods” section on DNA extracted from tissue and cultured fibroblasts of 7 patients with keloids. A typical pattern of p53 gene mutational analysis using nonradioisotopic PCR/SSCP with silver staining is shown in Figure 1.

DNA sequence analysis was performed for all samples that showed a positive PCR/SSCP mutational pattern. The results of the sequence analysis are listed in the Table. All 7 patients with keloids had detectable mutations in

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The p53 gene. These mutations were limited to exons 4, 5, and 6 and were maintained in cultured keloid fibroblasts; however, they were not present in either the normal (buccal smear) DNA or fibroblast cultures of healthy skin from the same patients. A mutation in exon 5 resulting in amino acid substitution was found in 1 patient in both keloid tissue and cultured keloid fibroblasts (codon 156, CGC→CCC, arginine→proline). Frameshift mutations in exons 5 and 6 caused by the insertion or deletion of a nucleotide at different positions were found in 6 patients in both keloid tissues and cultured fibroblasts. Mutations in exon 4 resulting in amino acid substitution were found in all patients in both keloid tissues and cultured fibroblasts (all in codon 72, CGC→CCC, arginine→proline). No mutations were detected in buccal smear DNA, healthy skin, or cultured fibroblasts from skin without lesions in any of the patients with keloids, indicating that these mutations were somatic. We identified codon 72 as a hotspot for p53 mutations in keloids (Figure 2).

**COMMENT**

Recent studies have implicated that the p53 tumor suppressor gene plays a critical role in the development of human cancers. Mutations in the p53 gene have been detected in approximately half of human cancers. While the importance of this gene with respect to tumor growth is not under debate, the question remains whether these mutations are the cause or the consequence of neoplastic growth. The p53 gene is known to exhibit distinct mutational patterns in various cancer types, which may reflect etiologic contributions of exogenous (environmental) and/or endogenous factors in the development of human cancers. There is increasing evidence that mutations in the skin p53 gene are among the most common somatic genetic alterations in human cancers. Germ line mutations at this locus can also lead to the development of Li-Fraumeni syndrome, a heritable cancer syndrome.

The detection of mutations in the p53 gene has been simplified by techniques such as PCR/SSCP and di...
The number of p53 mutations at various codons detected in keloid skin (KS) and keloid fibroblasts (KF) was obtained from the Table and plotted on a graph. Codon 72 is a hotspot for the p53 mutation.

rect DNA sequencing. Polymerase chain reaction/single-strand conformational polymorphism allows for the detection of a single-base change, since different single-stranded DNA assume distinct conformations and migration patterns. Polymerase chain reaction/single-strand conformational polymorphism is often used as a screening method for samples likely to have p53 mutations. DNA sequencing is then used to verify and further characterize the mutations as well as to pinpoint their locations.

We have used the PCR/SSCP technique followed by DNA sequencing analysis to detect p53 DNA mutations in tissues and cultured fibroblasts from 7 patients with keloids. Thirteen different mutations in the p53 gene were identified in these patients (Table). Eight of these mutations were characterized as point mutations in exon 4 or 5 that resulted in a proline-to-arginine substitution at codon 72 or 156. The proline-to-arginine substitution at codon 72 represents a common amino acid polymorphism in the p53 gene, the functional significance of which is unknown.28 However, this hotspot mutation for the p53 gene in keloid fibroblasts was not detected in normal fibroblasts. This excludes the possibility that this locus is for amino acid polymorphism and without functional significance. The proline-to-arginine substitution at codon 156 found in 2 patients is identical to that described in the p53 gene of a human osteosarcoma cell line.29

In 6 of the patients with keloids, the mutation in the p53 gene was a frameshift (nonsense) mutation that may lead to truncated or unstable p53 protein. In the biologic mechanisms of cancer, p53 mutations are spread throughout the gene and are mostly missense mutations, but most of these mutations are located in exons 4 through 9, which encode for highly conserved regions of the p53 protein.30 Detectable hotspot mutations for the p53 gene at codon 72 of keloid lesions and cultured fibroblasts suggests the production of a mutant protein that has increased cellular stability, since mutations in exons 4 through 9 have been most frequently associated with conformational changes in p53 protein in other malignancies.28 These findings are consistent with those of our previous study, in which we clearly demonstrated by immunohistochemistry that p53 mutant protein accu-

mulates in cultured keloid cell lines of up to 10 passages, while normal fibroblasts do not express detectable levels of p53 protein.31 Increasing the cellular stability of the p53 gene may increase its transcriptional activity, which in turn may lead to an altered balance between apoptosis and the proliferation of keloid fibroblasts. Further evidence that exogenous and/or environmental agents can have a significant impact on the regulation of fibroblast growth and differentiation was demonstrated by short-term exposure to doxorubicin, which was not cytotoxic, but appeared to augment normal fibroblast proliferation and p53 protein expression.31

The relationship between p53 and the pathway of apoptosis has been well characterized. Studies focusing on the effect of p53 gene mutations on both apoptosis and cell-cycle progression of keloid fibroblasts are ongoing in our laboratory. There are no data that link collagen production to p53 mutations in keloid fibroblasts. Studies to determine the effect of p53 mutations on the ability of keloid fibroblasts to overproduce or underdegrade collagen are also ongoing in our laboratory. Our preliminary data show that the rate of apoptosis is drastically reduced in keloid fibroblasts compared with that in normal fibroblasts. This may be due to the production of a mutant p53 protein that is not capable of inducing apoptosis in response to toxic environmental stimuli. Taken together, this work suggests that p53 gene mutation is a key regulator in the formation of keloids. However, such a finding does not eliminate other causal factors.3 We believe we have a model that will allow us to perform future studies of mechanisms by which p53 mutations contribute to the formation of keloids. In addition, our model will allow us to maintain long-term cultures of fibroblasts with p53 mutations and cultures of healthy skin without p53 mutations in patients with keloids.

The finding that the p53 gene is mutated in the keloid fibroblasts of all patients in our study has led us and may lead other investigators to develop an animal model to test the hypothesis that keloids are formed as a consequence of p53 mutations. This type of an animal model would be an invaluable tool for determining the molecular origin of keloidogenesis and evaluating new treatment strategies.

Accepted for publication February 20, 1998.

This study was supported in part by a departmental fund from the Department of Dermatology, Henry Ford Hospital.


The authors acknowledge E. Sarkar, PhD, Department of Pathology, Wayne State University, Detroit, Mich, for his kind advice and help throughout this study.

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Clinical Implications

Mutations of p53 have been implicated in a wide range of neoplasms. It is generally believed that mutations in this tumor suppressor gene lead to the uncontrolled proliferation of cells and dysregulation of apoptosis. In the present study, Saed et al found similar mutations in all of the keloids they examined. This observation raises several biologic questions and provides a direction for future therapeutic intervention. The finding of increased p53 mutations within these lesions may explain the dysregulated repair sequence followed by the fibroblasts in this condition. However, this finding also raises questions about the role of p53 in neoplasia in general and malignancy in particular. Keloids are known to be locally aggressive neoplasms, they are not known to metastasize. Thus, in this situation, it appears that p53 mutations may play a role in stimulating or permitting local growth, but are not sufficient to allow neoplasms to develop metastatic capability. If these assumptions prove to be correct, keloid treatment could someday be amenable to localized gene therapy. Intact p53 genes could be introduced into lesional fibroblasts, reintroducing normal cellular regulation into this disordered population of cells. It should be stressed, however, that these data are preliminary, and much more extensive work, probably with animal models, needs to be developed before these types of interventional techniques can be seriously considered.

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