Detection and Identification of Activated Oncogenes in Human Skin Cancers Occurring on Sun-exposed Body Sites

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ABSTRACT

High-molecular-weight DNA isolated from eight fresh human skin cancers occurring on sun-exposed body sites were assayed for their ability to transform NIH 3T3 cells. A cotransfection protocol using pSV2-neo DNA, which confers resistance to the antibiotic G418, was used to select cells that had taken up the transfected DNA. About 2 weeks after transfection, G418-resistant colonies were pooled and injected s.c. into athymic nude mice. The NIH 3T3 cells transfected with DNA from six of the human skin cancers induced tumors in nude mice. DNAs from all six tumor cell lines contained human alu sequences. Southern blot hybridization with ras-specific probes revealed that DNAs from the four ala-rich tumors contained the human Ha-ras oncogene, in addition to that of the NIH 3T3 controls. In contrast, DNAs from the other two tumors did not contain any of the known oncogenes tested, except those endogenous to NIH 3T3 cells. DNAs from three of the four first cycle tumorigenic transformants gave rise to morphologically transformed foci when assayed in a second cycle of transfection. DNAs from all three secondary transformants contained discrete human alu sequences, and in addition, contained Ha-ras sequences similar to those present in their respective primary transformants. Interestingly, DNA from both primary and secondary transformants of one particular human squamous cell carcinoma contained highly amplified copies of the Ha-ras oncogene. These results suggest that activation of the Ha-ras oncogene may be common in human skin cancers originating on sun-exposed body sites. Further characterization of the Ha-ras oncogenes present in these human skin cancers may provide information on the molecular mechanisms by which UV radiation of the sun induces human neoplasms on exposed body sites.

INTRODUCTION

Many cellular oncogenes are known to be associated with a variety of human and rodent tumors. In fact, induction of tumors in rodents by defined chemicals has frequently resulted in activation of specific oncogenes (1–8). About 20% of human tumors and chemically induced tumors contain activated ras genes (designated Ha-ras, Ki-ras, and N-ras) capable of inducing morphological and tumorigenic transformation when introduced into NIH 3T3 cells by DNA-mediated gene transfer (9–17).

Cellular oncogenes can be activated by both point mutations and chromosomal translocations, suggesting that there may be a direct link between exposure to agents that damage DNA and genetic change leading to malignancy (4, 6, 7, 18–20). Zarbl et al. (7) have shown that in rat tumors induced by nitrosomethylurea, the point mutation responsible for the malignant activation of the Ha-ras-1 locus was always at the 12th codon, whereas in rat tumors induced by DMBA, the point mutation was at the 61st codon of the Ha-ras-1 locus. Similarly, Quintanilla et al. (6) have reported that over 90% of tumors, including premalignant papillomas, induced in mice by DMBA as an initiator and tetradecanoylphorbol-13-acetate as a tumor promoter contained a specific A-T transversion in the second nucleotide of codon 61 of the Ha-ras gene. In addition, two non-ras oncogenes, termed met and neu, are reported to be expressed in a human cell line transformed by N-methyl-N-nitro-N-nitrosoguanidine and in a rat neuro/glioblastoma cell line derived from tumors induced by ethyl nitrosourea (21, 22).

One hypothesis suggested by these observations is that a particular carcinogen may activate specific oncogenes during the development of tumors. However, it is not known whether UVR activates protooncogenes in a carcinogen-specific manner. This question is of considerable importance because UVR is a potent DNA damaging agent and a known inducer of skin cancer in experimental animals. In addition, UVR present in sunlight is responsible for the induction of most skin cancers in humans (23–25).

There is one report which suggests that some UVR-induced skin cancers in mice express an activated Ki-ras oncogene (26). However, there are no reports available on activated oncogenes in nonmelanoma human skin cancers that originated on sun-exposed body sites. Since cancers of the skin are the most prevalent form of human cancer, it is important to determine whether they contain specific transforming genes. Therefore, we investigated whether DNAs from fresh human skin cancers occurring on sun-exposed body sites contained oncogenes capable of inducing morphological and tumorigenic transformation when introduced into NIH 3T3 cells by DNA-mediated gene transfer.

MATERIALS AND METHODS

Human Tumors. The primary human skin cancers used in this study were originated on sun-exposed body sites of the patients. The location of tumors, tumor types, and clinical evaluation of the patients are shown in Table 1. Fresh tumors, unexposed to prior chemotherapy or radiation, were obtained at the time of surgical resection and immediately frozen at −70°C until processed for DNA extraction. Normal skin tissue from one of the patients (patient 6) was obtained from a non-sun-exposed site (gluteus) by punch biopsy and was used as a control.

Preparation of Cellular DNA. High-molecular-weight genomic DNA was extracted by the method described by Wigler et al. (27), with slight modifications. Frozen tumor tissues were thawed, minced thoroughly on ice with dissection scissors, and washed twice with ice-cold phosphate-buffered saline. The minced tissues were resuspended in lysis buffer (1% SDS-150 mM NaCl-10 mM Tris-HCl, pH 8.0-10 mM EDTA) containing 200 μg/ml proteinase K. The viscous lysates were heated at 65°C for 15 min and then incubated overnight at 37°C. Equal volumes of 650 mM NaCl-10 mM EDTA-10 mM Tris-HCl (pH 8.0) were added to the lysates and extracted twice with an equal volume of buffered saturated phenol. The aqueous phase was extracted once with an equal volume of phenolchloroform:isoamyl alcohol (25:24:1) and once more with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase with two volumes of cold absolute ethanol. The precipitated DNA was recovered with a Pasteur pipet and washed successively with 2 changes of 70% ethanol and 2 changes of 100% ethanol. The DNA precipitate was air dried and dissolved in sterile 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Cellular DNAs were sized by gel electrophoresis in 0.8% agarose gels. Uncut and

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: DMBA, dimethylbenzanthracene; UVR, ultraviolet radiation; BCC, basal cell carcinoma; SDS, sodium dodecyl sulfate.
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RESULTS

The various human skin cancers used in this study are shown in Table 1. Seven of the tumors were diagnosed as poorly differentiated squamous cell carcinomas and one as moderately differentiated squamous cell carcinomas and one as BCC. The patients were evaluated clinically for history of skin exposure. Clinical evidence of sun exposure included the presence of one or a combination of the following: solar keratoses, BCC, pigmentation, telangiectasias, erythema, and actinic lentigo. All of the patients exhibited one or more of these signs. Based on these observations and other information obtained from interviews with each subject, we can conclude that UVR was probably responsible for the induction of skin cancers in these patients.

Cotransfection of NIH 3T3 cells with each human tumor, normal human skin, or calf thymus and pSV2-neo DNAs yielded 1500-2000 G418-resistant colonies among four culture dishes. In contrast, NIH 3T3 cells transfected with pSV2-neo DNA alone resulted in a 10-fold decrease in the number of G418-resistant colonies obtained. Thus, cotransfection with tumor or normal DNA as a carrier enhanced the frequency of G418-resistant colonies and also ensured that a majority of G418-resistant colonies obtained by the cotransfection protocol took up the carrier DNA.

The colonies from each dish were pooled and 5 × 10⁶ cells from such a pool were injected s.c. into each of four nude mice. The time of first appearance of tumors and their subsequent growth were noted. The results shown in Table 2 indicate that NIH 3T3 cells transfected with DNA from six human skin cancers (five squamous cell carcinomas and one BCC) induced progressively growing tumors in nude mice within 2-5 weeks of injection. DNA from two of the human skin cancers did not induce tumorigenic transformation of NIH 3T3 cells. Similarly, untransfected NIH 3T3 cells or NIH 3T3 cells cotransfected with normal human skin or calf thymus and pSV2-neo DNAs did not induce tumors within this time period. The tumor incidence and the latency period varied, depending upon the transfected DNA. Whereas NIH 3T3 cells transfected with DNA from tumors 1, 3, and 6 induced tumors in 100% of mice injected, with latency periods of 2-3 weeks, NIH 3T3 cells transfected with DNAs from human tumors 2, 4, and 5 induced tumors in only 25-50% of mice injected, with latency periods of 4-5 weeks.

All the cell lines established from tumors induced in nude mice by various DNA transfectants were found to be resistant to the antibiotic G418. DNAs prepared from these cell lines were analyzed for the presence of human repetitive sequences. Previous studies have shown that human DNA segments can be detected by sequence hybridization after they have been introduced into NIH 3T3 cells (10, 30, 33). Fig. 1 shows an analysis of the DNAs of cell lines established from representative tumors induced in nude mice by various human tumor DNA transfectants. Tumors induced in nude mice by DNA transfectants of tumors 1, 3, 5, and 6 contained numerous human repetitive alu sequences (Lanes b, d, f, and g); the other two cell lines contained very few human alu sequences (Lanes c and e). As expected, DNA from untransfected NIH 3T3 cells (Lane a) or NIH 3T3 cells cotransfected with calf thymus and pSV2-neo DNAs (data not shown) did not contain any human alu sequences.

In order to determine whether the tumors induced in mice by various human skin cancer DNA transfectants contained specific oncogenes, their cell line DNAs were analyzed for the presence of human Ha-, Ki-, and N-ras genes by Southern blot analysis. Newly acquired ras genes in mouse tumors induced by human tumor DNAs can be detected either by finding additional restriction fragments or by comparing band intensities with appropriate controls on Southern blots. The hybridization pattern observed with the N- and Ki-ras probes in mouse tumors induced by various human tumor DNAs was similar to that with untransfected NIH 3T3 cells (data not shown), indicating that these tumorigenic transfectants did not contain the human N- or Ki-ras oncogenes. However, when duplicate blots were hybridized with the Ha-ras probe, the cell lines from tumors induced in mice by injection of NIH 3T3 cells transfected with...
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Table 1  
Origin and type of human skin cancers used in DNA transfection  

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor location</th>
<th>Tumor type</th>
<th>Clinical observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>53</td>
<td>Upper lip</td>
<td>SCC</td>
<td>Numerous SCC, BCC, and actinic keratosis</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>56</td>
<td>Right ear</td>
<td>BCC</td>
<td>Numerous SCC and BCC</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>51</td>
<td>Upper lip</td>
<td>SCC</td>
<td>Few SCC and BCC</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>69</td>
<td>Neck</td>
<td>SCC</td>
<td>Few SCC and BCC</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>60</td>
<td>Left ear</td>
<td>SCC</td>
<td>Numerous SCC, BCC, and actinic keratosis</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>61</td>
<td>Left temple</td>
<td>SCC</td>
<td>Numerous SCC, BCC, and actinic keratosis</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>78</td>
<td>Left ear</td>
<td>SCC</td>
<td>Numerous SCC, BCC, and actinic keratosis</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>78</td>
<td>Left ear</td>
<td>SCC</td>
<td>Numerous SCC, BCC, and actinic keratosis</td>
</tr>
</tbody>
</table>

All the patients were white Caucasians.  
* SCC, squamous cell carcinoma.

Table 2  
In vivo tumorigenicity of cotransfected NIH 3T3 cells  

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Time to 1st tumor (wk)</th>
<th>Mean tumor diameter (mm) at 6 wk</th>
<th>Tumor incidencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor 1 + pSV2-neo</td>
<td>2</td>
<td>22.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Tumor 2 + pSV2-neo</td>
<td>4</td>
<td>17.6</td>
<td>4/4</td>
</tr>
<tr>
<td>Tumor 3 + pSV2-neo</td>
<td>3</td>
<td>18.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Tumor 4 + pSV2-neo</td>
<td>5</td>
<td>6.8</td>
<td>1/4</td>
</tr>
<tr>
<td>Tumor 5 + pSV2-neo</td>
<td>4</td>
<td>17.0</td>
<td>2/4</td>
</tr>
<tr>
<td>Tumor 6 + pSV2-neo</td>
<td>2</td>
<td>27.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Tumor 7 + pSV2-neo</td>
<td>0</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Tumor 8 + pSV2-neo</td>
<td>0</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Normal skin + pSV2-neo</td>
<td>0</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0/4</td>
<td></td>
</tr>
</tbody>
</table>

Number of mice with tumor/number of mice given injections.  
Mean tumor diameter at 5 weeks.  
From patient 6.

Fig. 1. Detection of human alu sequences in cell lines from tumors induced in nude mice by NIH 3T3 cells transfected with DNA from various human skin cancers. DNAs (10 μg each) were digested with EcoR I and analyzed by Southern blot hybridization to 32P-labeled alu probe (Palu.1, Oncor, Inc.) under conditions of high stringency. Lane a, NIH 3T3; Lanes b–g, tumors induced by DNA from the following human skin cancers: tumor 1 (Lane b), tumor 2 (Lane c), tumor 3 (Lane d), tumor 4 (Lane e), tumor 5 (Lane f), tumor 6 (Lane g). Numbers on the ordinate are HindIII-digested λ-phage DNA molecular weight (kilobase) markers. The expected human Ha-ra,v band is 6.6 kilobases for EcoRI.

DNAs from tumors 1, 3, 5, and 6 exhibited additional bands besides the mouse endogenous Ha-ros sequences (Fig. 2). These newly acquired restriction fragments most likely represented activated forms of the human Ha-ros oncogene. Interestingly, mouse tumors induced by DNA transfectants of tumor 6 contained highly amplified copies of the Ha-ros gene. In contrast, DNA from tumors induced in mice by DNA transfectants from tumors 2 and 4 did not exhibit additional restriction fragments or increased intensity of hybridization with the Ha-ros probe. Furthermore, no homologies were detected with mos, myc, fos, fes, src, sis, abl, or erbB probes except those sequences that are endogenous to the NIH 3T3 cell genome (data not shown).

Since Ha-ros gene was found to be transmitted from human skin cancers to NIH 3T3 cells via DNA transfection, we analyzed DNAs from original human skin cancers for possible rearrangements and/or amplifications in the Ha-ros gene. The results shown in Fig. 3 reveal that human skin cancers 1, 3, 4, and 6, as well as the normal skin tissue from patient 6, contained the typical single 6.6-kilobase BamHI fragment homologous to the Ha-ros gene. In contrast, the Ha-ros gene present in human skin cancers 5, 7, and 8 appeared to be rearranged, and in one case also amplified (tumor 5). Nothing can be said about human tumor 2 because there was not enough DNA available for analysis. These results suggest that transformation of NIH 3T3 cells by DNAs from human skin cancers 1, 3, and 6 could be due to mutations in the Ha-ros gene, whereas transformation by DNA from human tumor 5 could be due to rearrangement and/or amplification of the Ha-ros gene.

It is very likely that Ha-ros gene was responsible for tumorigenic transformation of NIH 3T3 cells in the primary transfection because each nude mouse tumor was induced by a single transfectant receiving 1/1000 of a genome equivalent (30).
of this assay we can ascertain whether the Ha-ras gene detected from patient 6 failed to induce transformed foci (0.008 foci/μg DNA).

Representative foci were isolated and purified by growing in semisolid agar. Interestingly, a single focus found in each of the secondary transfectants of human tumor 5 and primary transfectants of normal human skin tissue from patient 6 did not produce colonies in soft agar. DNAs were extracted from cloned cells and analyzed for the presence of human alu and Ha-ras sequences. All three secondary transformants of human skin tumors 1, 3, and 6 contained discrete human alu sequences (Fig. 4). These conserved alu sequences are probably tightly linked to the Ha-ras gene in the original human skin cancers. Southern blot analysis with a Ha-ras probe revealed that DNAs from secondary transfectants of human tumors 1 and 6 contained the unique 9.4-kilobase BamHI fragment of the Ha-ras gene similar to those found in their respective primary transfectants, whereas the secondary transfectants of human tumor 3 contained the 6.6-kilobase BamHI fragment of the Ha-ras gene similar to that found in its primary transformants (Fig. 5). Furthermore, the Ha-ras gene in secondary transformants of human tumor 6 was again highly amplified analogous to its primary transformants. Thus, these results suggest that a stable, activated Ha-ras oncogene was being transferred from all three human skin cancers to NIH 3T3 cells.

DISCUSSION

In this report, we examined eight human skin cancers occurring on sun-exposed body sites for the presence of oncogenes detectable by DNA transfection. Using a cotransfection and tumorigenicity assay in nude mice, we could detect the transfer of oncogenes from four of eight human skin cancers into NIH 3T3 cells. However, DNAs from only three of four primary tumorigenic transformants induced morphologically transformed foci in a second round of transfection. The transforming the other hand, it is quite possible, at least in some cases, that tumorigenic transformation was really not due to Ha-ras gene, but due to some other gene near Ha-ras. Therefore, we performed a second round of transfection, using DNAs from primary transformants to determine whether the Ha-ras gene detected in four human tumors could be passaged serially. A NIH 3T3 focus assay was used instead of the cotransfection and tumorigenicity assay because this assay, when used in second or third round of transfection, preferentially detects Ha-ras genes containing mutations (18, 19). Therefore, by means of this assay we can ascertain whether the Ha-ras gene detected in the primary transformants of four human tumors contains structural alterations. As shown in Table 3, DNAs from primary transformants of human tumor 1, 3, and 6 induced morphologically transformed foci at frequencies of 0.2, 0.15, and 0.4 foci/μg DNA, respectively. In contrast, DNA from primary transformants of human tumor 5, and DNA from normal skin tissue.
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Fig. 5. Presence of Ha-ras sequences in secondary transformants. DNAs were extracted from soft agar-purified secondary transformants and an aliquot (10 µg each) was digested with BamHI and analyzed by Southern blot hybridization to 32P-labeled Ha-ras probe. Lane a, NIH 3T3; Lanes b, c, and d are secondary transformants of human tumor 1, 3, and 6, respectively. Numbers on the ordinate are molecular weight markers in kilobases.

genes present in primary and secondary transformants of these tumors were identified as the Ha-ras gene. These results suggest that the Ha-ras gene present in the three human skin cancers may contain structural mutations. The failure by primary transformants of human skin tumor 5 to induce morphologically transformed foci in a second round of transfection could be due to the possibility that the Ha-ras gene in human tumor 5 may not contain a point mutation. It is possible that the transforming activity of human tumor 5 DNA, as detected by cotransfection and tumorigenicity assays, could be due to Ha-ras gene amplification and/or rearrangement. This possibility is supported by the fact that the Ha-ras gene present in the original human tumor 5 DNA appears to be amplified, as well as, slightly rearranged (Fig. 3). Alternatively, the gene detected in primary transformants of human skin cancer 5 was not really a Ha-ras gene, but some other gene near Ha-ras. In any event, these results indicate that the nude mouse tumorigenicity assay may be more sensitive to transformation by amplified Ha-ras genes or other oncoproteins than the NIH 3T3 focus assay.

Interestingly, the Ha-ras gene was found to be greatly amplified in both the primary and secondary transformants of human tumor 6. This amplification might have occurred during cotransfection procedure, because the original human tumor 6 did not contain amplified copies of the Ha-ras gene. As we reported earlier (34), both the primary and secondary transformants induced by human tumor 6 DNA (reported earlier as tumor AS) not only produced s.c. tumors at the site of injection but also metastasized spontaneously to the lungs in 100% of mice injected.

DNAs from two human skin cancers were weakly tumorigenic, whereas DNAs from the remaining two human skin cancers were nontumorigenic in the NIH 3T3 tumorigenicity assay. The tumors induced in nude mice by DNA transformants of skin cancers 2 and 4 contained very few human alu sequences as compared to the tumors induced by DNA transformants of other human skin cancers. Furthermore, DNAs from tumors induced by DNA transformants of human skin cancers 2 and 4 did not reveal the presence of human Ha-ras, Ki-ras, N-ras, mos, myc, fes, fos, src, sis, abl, or erbB oncoproteins. However, the possibility that these tumors contained some other oncoproteins cannot be ruled out.

The incidence of transfection-positive human skin cancers was found to be slightly higher (37%) than that observed with other human or rodent tumors. This high incidence may be attributable to the fact that the NIH 3T3 “tumor assay” used by us and others (30, 34-36) may be more sensitive than the NIH 3T3 focus assay for detecting transforming genes. The vast majority of the DNA samples from human or rodent tumors do not have transforming activity in the NIH 3T3 focus assay; only about 20% of human tumors and chemically induced rodent tumors are capable of inducing transformed foci (10, 11, 15, 37). One possible explanation for this observation could be that the many genetic elements responsible for tumor formation cannot effect morphological transformation of NIH 3T3 cells. On the other hand, the high frequency of transfection-positive human skin cancers that we observed could be due to a statistical error, i.e., small sampling size. If we had analyzed a large number of samples, the frequency of transfection-positive human skin cancers might have been lower than the 37% we observed.

Even though our studies demonstrated that three of eight human skin cancers occurring on sun-exposed body sites contained Ha-ras oncogenes capable of inducing morphological and tumorigenic transformation of NIH 3T3 cells, we do not know the nature of the mutations responsible for the malignant activation of the Ha-ras oncogene in these human skin cancers. Recent studies have shown that a majority of the tumors induced in rats and mice by chemical carcinogens such as DMBA and NMU contain specific ras gene mutations (3, 6-8). Mutations at a particular Ha-ras gene locus appear to be correlated with the type of carcinogen used to induce the tumors. It will be interesting to determine whether the Ha-ras oncogene activated in the three transfection-positive human skin cancers contain specific mutations that are unique to tumors induced by UVR. Further characterization of the activated Ha-ras gene in human skin cancers occurring on sun-exposed body sites and its comparison with the reported mutations of the Ha-ras gene in tumors induced by specific chemical carcinogens may provide insights into the ways in which UVR can activate protooncogenes in human skin cancers.

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REFERENCES

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