Transforming Potential Is Detectable in Arteriosclerotic Plaques of Young Animals

Arthur Penn, Frank C. Hubbard Jr., and Joan Lee Parkes

The carcinogen-treated cockerel is a model for studying the early stages of arteriosclerotic plaque development. Carcinogen administration accelerates arteriosclerotic plaque development in cockerels, and transforming elements are present in DNA from advanced human plaques. In this study, we asked whether transforming elements could also be detected at early stages of plaque development in cockerels. NIH3T3 cells were transfected with DNA from plaques isolated from carcinogen-treated cockerels and from the healthy arterial wall underlying the plaques. Approximately $5 \times 10^6$ cells from each group were injected into nude mice. Tumors appeared in five of five mice in the plaque DNA group; no tumors appeared in mice from the healthy arterial wall group. All five plaque DNA-associated tumors hybridized to a cockerel genomic probe. Eight cockerel-specific bands were identified in EcoRI digests of first-round (primary) tumors. DNA from a primary tumor was tested in a second round of transfection. Five of five mice developed tumors after injection with these secondary transformants. All second-round tumors contained cockerel DNA, and a prominent cockerel-specific band (>28 kb) was seen in EcoRI digests of all second-round tumors. In addition, a 5.2-kb band appeared prominently in one of five second-round tumors. No evidence was found for activation of the oncogenes Ha-ras, Ki-ras, src, or myc in the plaque-associated tumors. Similarly, DNA from plaque-associated tumors did not hybridize to probes for Marek disease virus, herpes simplex virus 1, or reverse transcriptase, suggesting that neither herpesviruses nor retroviruses are involved in the transforming activity of plaque DNA. These results indicate that transforming elements are a general property of arteriosclerotic plaques and are detectable in plaques of young animals.

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The proliferation of smooth muscle cells (SMCs), a rare event in the normal adult arterial intima, is thought to be critical to the development of arteriosclerotic plaques. Most theories of plaque development have regarded SMC proliferation as a reactive process, occurring in response to such stimuli as injury or inflammation. Under these conditions the SMCs involved in plaque formation are regarded as indistinguishable (except for receipt of stimuli that cause proliferation) from the rest of the arterial wall SMCs. According to this view, it is the availability of mitogens or other modulators of cell division rather than any unique property of the responding arterial SMCs that determines whether intimal SMC proliferation and subsequent plaque development will occur.

A fundamentally different view is presented by the monoclonal hypothesis.3 This proposes that the proliferating SMCs are derived from a stably transformed and thus permanently altered cell population that is distinct from the bulk of arterial SMCs. One inference from this hypothesis is that there should be demonstrable similarities, on the molecular level, between events associated with the development of both plaques and tumors. Indeed, the prediction has been made that mutagens and viruses should play a role in plaque formation comparable to the role that they play in tumor formation. Studies including those using cockerels as an animal model have provided indirect support for this view. Weekly injections of polynuclear aromatic hydrocarbon carcinogens (PAHCs) at nontumorigenic doses into cockerels markedly accelerate plaque development.4–7 The inducible enzyme systems responsible for the metabolism of PAHCs have been identified in the artery wall of both cockerels and mammals.8–10 The PAHC 7,12-dimethylbenz[a]anthracene (DMBA), administered as an “initiator” followed by weekly injections of the $\alpha$-adrenergic agonist methoxamine (as a promoter) yielded microscopic aortic plaques in cocker-
els.11 A series of studies during the past decade have also suggested a role for herpesviruses in plaque formation.12–20 Two recent sets of in vitro studies have provided evidence for possible mechanisms of the interaction between viruses and SMCs. In the first of these studies fibroblast growth factor receptor was identified as the site of herpesvirus entry into mammalian cells, including SMCs.21 In the second study rabbit aortic SMCs were transformed by the early region of SV40 DNA.22

Carcinogen and virus studies have thus far provided only indirect support for the predictions of the monoclonal hypothesis. Direct support requires demonstration of molecular alterations in plaque cells comparable to those observed in tumor cells. Earlier, we reported that human coronary artery plaque DNA contained transforming potential and that mouse fibroblasts (NIH3T3 cells) transfected with human coronary artery plaque DNA gave rise to tumors after subcutaneous injection into nude mice.23 The protocols followed in these studies were originally developed to demonstrate the presence of dominant transforming genes (oncogenes) in tumors and transformed cell lines.24–26

One limitation associated with these human studies is that the plaques were obtained from older patients in whom atherosclerosis developed over a period of decades. Thus, it could be argued that acquisition of transforming potential is a late-stage event in atherosclerosis and is only indirectly associated with plaque etiology.

An advantage of using the carcinogen-treated cockerel as a model for plaque development is that large plaques can be generated in the abdominal aortas of these young animals after carcinogen treatment. These plaques are fibromuscular, with limited lipid involvement and without apparent calcification or necrosis. We have shown that in this system carcinogen treatment acts primarily to accelerate plaque development rather than to initiate it.4–7 Morphological and ultrastructural similarities between fibromuscular abdominal aortic plaques in cockerels and coronary artery plaques in humans have been documented.27 The objective of the present studies was to determine whether molecular alterations exist in carcinogen-associated cockerel plaques similar to those previously identified in human plaques.23

In this article we report that NIH3T3 cells are transformed by carcinogen-associated cockerel plaque DNA and that injection of these transformed cells into nude mice elicits development of tumors that contain cockerel genomic DNA. Moreover, this plaque transforming potential is transmitted serially. Thus, there are molecular alterations in the DNA of plaques from diverse sources (human and avian) and from young and old subjects that are similar to alterations that have been identified in tumor DNA. This suggests that somatic cell mutations play a role in plaque etiology.

Methods

Animals

Four-week-old white leghorn cockerels (Kerr Hatcheries, Hightstown, N.J.) were housed in stainless steel cages. Water and standard mash (Purina Co., St. Louis, Mo.) were available ad libitum. All animal handling and treatment procedures were conducted according to New York University Medical Center guidelines. From 6–22 weeks of age the cockerels received weekly injections of DMBA (20 mg/kg body wt, Sigma Chemical Co., St. Louis, Mo.) into the pectoral muscle. The DMBA was dissolved in dimethyl sulfoxide (Fisher Chemical Co., Valley Forge, Pa.). After the cockerels were humanely killed at 23 weeks of age, plaques and healthy arterial wall underlying the plaques were removed under sterile conditions and frozen immediately in liquid N2.

Transfections

All tissue samples were pulverized in liquid N2.23 DNA extraction was performed via standard phenol/CHCl3/isoamyl alcohol procedures.28 DNA from four separate sources was tested in the transfection assay: 1) cockerel aortic plaque, 2) healthy cockerel arterial wall that had underlain the plaques, 3) T24 human bladder carcinoma cell line (positive transfection control), and 4) NIH3T3 cells (negative transfection control). The DNAs were cotransfected with pSV2neo29 the hybrid plasmid vector conferring antibiotic resistance, by the standard Ca3(PO4)2 transfection protocol.30 For each sample 40 µg genomic DNA was transfected along with 4 µg pSV2neo into three T-25 flasks, each containing approximately 0.5 x 106 NIH3T3 cells grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Twenty-four hours later cells were split 1:3. Transfected cells were grown in the presence of gentamicin (G418).

Nude Mouse Assay

Three weeks after cotransfection the colonies were collected, and 4–5 x 106 cells from each group were injected subcutaneously into the scapular area of five 4–5-week-old athymic (nu/nu) female mice (Haran Sprague-Dawley, Indianapolis, Ind.). Animals were checked for the presence of tumors three times per week. The mice were killed when the tumors were at least 15 mm in diameter, and samples of tumors were taken for histological observation, DNA isolation, and growth in culture.

Southern Blot Hybridization

DNA was digested with the restriction enzymes EcoRI and BamHI according to the manufacturers' instructions, subjected to electrophoresis overnight in 0.7% agarose (Bio-Rad, Richmond, Calif.) at 20 V, 15 mA per gel, and blotted onto nitrocellulose31 (Schleicher and Schuell, Keene, N.H.). pSV2neo was provided by E. Newcomb, New York University Medical Center. The probes Ha-ras, Ki-ras, src, and myc, consisting
TABLE 1. Tumorigenic Efficiency of Plaque DNA-Transfected Cells

<table>
<thead>
<tr>
<th>DNA source</th>
<th>No. tumors/No. mice</th>
<th>Tumor latency (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteriosclerotic plaques from DMBA-treated cockerels</td>
<td>5/5</td>
<td>33-45</td>
</tr>
<tr>
<td>Healthy arterial wall (underlying plaque) from DMBA-treated cockerels</td>
<td>0/5</td>
<td>...</td>
</tr>
<tr>
<td>T24 cells (human bladder carcinoma cell line)</td>
<td>5/5</td>
<td>7-10</td>
</tr>
<tr>
<td>NIH3T3 cells</td>
<td>0/5</td>
<td>...</td>
</tr>
<tr>
<td>Plaque-associated primary tumor CT1-3 (from first source above)</td>
<td>5/5</td>
<td>24-42</td>
</tr>
<tr>
<td>T24 cells</td>
<td>5/5</td>
<td>19-23</td>
</tr>
<tr>
<td>NIH3T3 cells</td>
<td>0/5</td>
<td>...</td>
</tr>
</tbody>
</table>

For each group, ~5x10⁶ cells were injected subcutaneously in the dorsal surface of five female nu+/nu+ mice.

DMBA, dimethylbenz[a]anthracene.

of inserts cut out and purified from plasmids, were purchased from Oncor, Gaithersburg, Md.; the reverse transcriptase (RT) probe, contained in plasmid pRT 432-1, No. 31990, was purchased from the American Type Culture Collection, Rockville, Md. The herpes simplex virus 1 (HSV-1) probe was a gift of David Hajjar, Cornell University Medical Center, New York. The Marek disease virus (MDV) probe (BamHI-H insert), which does not hybridize to DNA from uninfected chicken cells, was provided by K. Schat, Cornell University, Ithaca, N.Y. The cockerel genomic probe was prepared from cockerel heart DNA. All the probes were labeled with phosphorus-32-labeled probe prepared from cockerel heart DNA. Hybridizations were performed under high stringency conditions (50% formamide, 43°C). Sources of DNA were lane 1, T24-associated nude mouse tumor; lane 2, NIH3T3 cells; lane 3, first-round cockerel plaque DNA-associated nude mouse tumor (CT1-3); lane 4, CT1-3 primary explant; lane 5, second-round nude mouse tumor (CT2-3); lane 6, second-round nude mouse tumor (CT2-4). Note that faint bands present in the T24-derived tumor and in the NIH3T3 cells (lanes 1 and 2) are most likely due to cross hybridization of the cockerel probe to mammalian repetitive sequences with which cockerel repetitive sequences share homology. Numbers at left are in kb.

Results

Cockerels were injected weekly with DMBA from 6–22 weeks of age and were killed at 23 weeks of age. Four of five DMBA-injected cockerels displayed grossly visible plaques (12x3 mm) in the distal third of the abdominal aorta. These plaques were similar to DMBA-associated plaques that we have described previously⁴⁻⁷; they were fibromuscular with little lipid involvement. DNA samples obtained from the pooled plaques, healthy arterial wall underlying the plaques, T24 human bladder carcinoma cell line, and NIH3T3 cells were used to cotransfect host NIH3T3 cells. Results of the transfection–nude mouse study are presented in Table 1. Tumors developed in all five mice injected with plaque DNA–transfected cells. The tumor latency period was 33–45 days. In contrast, no tumors appeared in any mice injected with cells transfected with DNA from normal arterial wall or from untreated NIH3T3 cells. All five mice in the T24 carcinoma cell line group developed tumors, with a latency period of 7–10 days. Tumors were excised when they reached 15–20 mm in diameter. All tumors had the appearance of poorly differentiated fibrosarcomas. DNA from all first-round plaque-associated tumors hybridized to a cockerel genomic probe. At least eight distinct cockerel-specific bands were visible in first-round plaque-associated tumors (see Figure 1).

DNA extracted from one of the plaque DNA–associated tumors (CT1-3) was tested in a second round of transfection. All five mice injected with the second-round transfectants developed tumors. Latency periods ranged from 24 to 42 days (Table 1). The positive control group in this study displayed tumors (five of five mice) after 19–23 days. Again, no tumors appeared in the negative controls.

DNAs isolated from the secondary tumors were digested with EcoRI and hybridized to a cockerel genomic probe. All secondary tumors contained cockerel genomic DNA. A prominent band (>28 kb) was identified in all secondary tumors. EcoRI digests
of two of these tumors are shown in Figure 1 (lanes 5 and 6). In addition, a 5.2-kb band appeared prominently in one of five second-round tumors (Figure 1, lane 6). As expected, neither DNA from the T24-associated tumors nor that from NIH3T3 cells hybridized to the cockerel genomic probe (Figure 1, lanes 1 and 2).

DNA from the first round of plaque-associated tumors was tested for the presence of activated oncogenes. Ki-ras, Ha-ras, myc, and src probes were used to screen BamHI-digested DNA from plaque-associated tumors. None of these was responsible for the transforming potential of cockerel atherosclerotic plaque DNA (Figure 2 and data not shown). In Figure 2 the intense band (>9.4 kb) in lane 1 (cockerel heart DNA) is cockerel myc; the less intense bands of approximately 6.6 kb in lanes 2–5 are murine myc. The absence of a >9.4-kb cockerel myc signal in the plaque-associated nude mouse tumors indicates that myc is not the cockerel plaque-transforming gene.

We also tested the possibility that retroviral infection plays a role in cockerel atherosclerotic plaque development. DNA from plaque-associated tumors was hybridized to an RT probe. No RT-specific bands were identified (data not presented).

Because a single injection of the avian herpes virus MDV to 4-day-old cockerels results in focal plaque formation, we screened the plaque-associated tumor DNA with MDV. This probe failed to hybridize to plaque-associated tumor DNA (data not presented). Finally, because herpesviruses have been implicated as etiologic factors in the pathogenesis of human atherosclerosis and human HSV-1 has been shown to influence lipid accumulation and metabolism in both human and bovine arterial SMCs, we also screened the plaque-associated tumor DNAs with an HSV-1 probe. This, too, was negative, suggesting that herpes viral sequences are not associated with the transforming element(s) in cockerel plaque DNA.

Discussion

Previously, we have shown that carcinogen treatment accelerates plaque development in cockerels. We have also demonstrated, via the transfection–nude mouse tumor assay, that human coronary artery plaque DNA has transforming potential. Recently, we reported that DNA from SMC strains derived from human aortic plaques is also positive in the transfection–nude mouse tumor assay.

The data presented here show that cockerel plaque DNA, like human plaque DNA, contains transforming element(s). NIH3T3 cells transfected with cockerel plaque DNA gave rise to nude mouse tumors in five of five injected mice. The tumor latency period (33–45 days) was significantly shorter than that for human coronary artery plaque–associated tumors (50–112 days) but longer than that for the T24-associated tumors (7–10 days), which contain an activated Ha-ras oncogene. As is the case with human coronary artery plaque DNA, cockerel plaque DNA transforming elements are serially transmitted, with DNA from primary plaque-associated tumors giving rise to secondary plaque-associated tumors (Table 1).

DNA from both rounds of tumors hybridized to a cockerel genomic probe. A number of cockerel-positive bands were present in EcoRI-digested DNA from plaque-associated first-round tumors and their primary explants (Figure 1). When DNA from one of the plaque-associated tumors was tested in a second round of transfection, a prominent cockerel-positive band (>28 kb) was retained in all the resulting tumors (Figure 1). In addition, a 5.2-kb band was present in one of the second-round tumors. The retention of cockerel-positive bands in the second-round tumors suggests that the putative transforming gene is associated with these sequences. We are currently investigating this phenomenon.

One approach to identifying the transforming gene in cockerel plaque DNA is to screen the plaque-associated tumor DNA with oncogene probes. We tested for the presence of an activated cockerel Ha-ras gene because mutations in codons 12 and 61 of Ha-ras have been reported in skin cells transformed by the PAHs benzo[a]pyrene (B[a]P) and DMBA, respectively, and because B(a)P and DMBA markedly accelerate plaque development in cockerels. However, we found no evidence of cockerel Ha-ras in any of the plaque-associated tumors even though the plaques were obtained from DMBA-treated cockerels. Similarly, we found no evidence for the presence of exogenous Ki-ras, src (not shown), or myc (Figure 2) in any of the cockerel plaque–associated nude mouse tumors. Ki-ras is one of the most commonly activated dominant transforming genes that has been identified in human tumors. Activation of src, which is responsible for the transforming activity of Rous sarcoma virus, results in malignant transformation of avian cells. myc was
first identified as the transforming gene of avian myelocytomatosis virus. In humans, enhanced expression of \( \text{myc} \) has been noted in cases of leukemia, lymphoma, and carcinoma of the breast and prostate. A modest elevation of \( \text{myc} \) expression was also observed in SMCs of spontaneously hypertensive rats compared with normotensive rats. We have recently described a twofold to sixfold enhancement of \( \text{myc} \) expression in SMC strains derived from human abdominal aortic plaques compared with healthy human aortic SMCs, although \( \text{myc} \) was not responsible for the transforming potential of the DNA from these cell strains.

Finally, the absence of RT, MDV, and HSV-1 sequences in the plaque-associated nude mouse tumors suggests that these viruses are not involved in the etiology of these cokerbel plaques. Although one recent report failed to identify transforming activity in human carotid plaques, other investigators have confirmed that serially transmitted transforming activity is present in human arteriosclerotic plaques (Reference 43 and R.M.L. Zwijnen, personal communication).

The results described herein confirm our previous findings with plaque samples of human origin and demonstrate that the presence of transforming elements is a general characteristic of plaque DNA. Furthermore, the fact that these activated elements exist in animals only 6 months old demonstrates that transforming potential is detectable in plaques of young animals.

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