Evidence for the progressive nature of neoplastic transformation in vitro

(chemical carcinogenesis/Syrian hamster/anchoragc independence/fibrinolytic activity)

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ABSTRACT The temporal acquisition of in vitro phenotypes associated with neoplasia was examined after exposure of Syrian hamster embryo cells to a chemical carcinogen. Quantitative assays measuring morphological changes, enhanced fibrinolytic activity, and anchorage independent growth were used to detect the development of transformed cells within a population of normal hamster embryo cells. Morphological transformation and enhanced fibrinolytic activity were early changes observed after treatment with benzo[a]pyrene, whereas the ability to grow in semisolid agar was delayed 32-75 population doublings after carcinogen exposure. This delay was not due to selection of a small number of cells that were present early after treatment but at a level below detection, because a large percentage of the cells isolated at early passage (10⁵-fold above the level of detection) developed the potential for anchorage independent growth at later passages. This development of the anchorage independent growth phenotype was induced by the carcinogen treatment, because spontaneous transformation was rare. These observations suggest that multiple cellular changes are required for the acquisition of the capacity for anchorage independent growth and that neoplastic transformation in vitro is a progressive process through qualitatively different stages. Thus, an analogy can be drawn to the progressive nature of in vivo carcinogenesis. These results strongly justify the study of oncogenesis in cell culture as a model for neoplastic transformation in vivo.

The concept of progression as defined by Foulds is one of stepwise neoplastic development through qualitatively different stages (1, 2). The progressive nature of a number of in vitro malignancies has been documented (1, 2). Since neoplastic transformation in vitro is assumed to reflect neoplastic development in vivo, the demonstration of progression during in vitro transformation is important in illustrating a parallel between in vitro and in vivo carcinogenesis.

Syrian hamster embryo cells in culture provide a useful model system for studying neoplastic transformation in vitro because they are stably diploid, have a low incidence of spontaneous transformation, and can be induced by chemical carcinogens to undergo neoplastic transformation (3-6). In addition to acquiring malignant potential, transformed hamster cells exhibit several other in vitro phenotypic characteristics often associated with neoplasia. These altered phenotypes can serve as useful markers in studying cell transformations. The detection of the phenotypes, however, requires quantitative assays with sensitivities sufficient to detect a few transformed cells within a population of normal cells.

Morphological transformation (4), enhanced fibrinolytic activity (7-9), and growth in soft agar (10-12) were the cellular changes chosen for study. Syrian hamster embryo cells were treated with benzo[a]pyrene (BaP) and the temporal acquisition of each of these altered characteristics was found to be independent (13). Morphological alterations were the earliest changes, followed by enhanced fibrinolytic activity; both of these phenotypes were expressed within 2 weeks after treatment, but were not necessarily correlated (13). Cells capable of growth in soft agar were not detected until a much later time. Evidence is presented in this paper that this delay in the expression of anchorage-independent growth indicates that neoplastic transformation in vitro is progressive in nature, involving multiple developmental stages.

MATERIALS AND METHODS

Cells. Syrian hamster embryo cell cultures were established from 13-day-gestation fetuses from inbred Syrian hamsters, as described (13, 14).

Assays for Transformed Phenotypes. Morphological transformation (as illustrated in Fig. 1) was scored as the number of transformed colonies per total surviving colonies by using described criteria (4, 5, 15). Enhanced fibrinolytic activity of individual transformed colonies was measured quantitatively by the appearance of clear zones of lysis in a fibrin/agarose overlay above the colonies (13).

Colony formation in agar was measured as described (11-13). Colonies containing over 25 cells were scored. Experiments with different transformed cell lines demonstrated that this method can detect 1 or 2 transformed cells in the presence of 10⁶ normal cells (Table 1).

For tumorigenicity studies, 2 x 10⁶ cells were injected subcutaneously into nonimmunosuppressed neonatal Syrian hamsters (Lakeview). Animals were observed for a period up to 1 yr.

Transformation Studies. Tertiary passage hamster cells were plated at 5 x 10⁵ cells per 75 cm² flask, allowed to attach overnight at 37°C, and then treated with BaP for 24 hr (13). Control cultures were treated with solvent only (0.1% dimethylsulfoxide). After treatment, all cultures were washed and incubated until nearly confluent. The cultures were then trypsinized and subcultures of 1-5 x 10⁶ cells were initiated. Cells from the cultures were also plated at low density for cloning (5-10 x 10⁵ cells per 100 mm petri plate) for 7-8 days of growth to examine resulting colonies for morphological transformation and fibrinolytic activity. At each passage, 200-600 colonies from each culture were scored for morphological transformation. 400-1000 colonies were examined for fibrinolytic activity, and 10⁶ cells were tested for growth in semisolid agar. The passages are numbered from the first passage after treatment, called post-treatment passage number one (PTP-1). This process was repeated at each PTP. The cells were passaged at confluence, every 6-9 days. Since the first subcult.

Abbreviations: BaP, benzo[a]pyrene; PTP, post-treatment passage.
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FIG. 1. Normal and transformed colonies. The periphery of a normal (left) and of a transformed (right) colony of Syrian hamster embryo cells. Stained with 10% aqueous Giemsa. (×40.)

ture occurred 1 week after treatment and was followed by cloning of the cells for 1 week, the colonies at PTP-1 were examined 2 weeks after treatment. In other experiments (13), single cells were plated at low density, treated for 24 hr with solvent only or carcinogen, and allowed to form colonies for 8 days without trypsinization (PTP-0).

Isolation of Colonies from the Fibrin/Agarose Overlay. Twenty-four hours after overlaying an 8-day-old colony with the fibrin/agarose, the cells were removed by physical manipulation with a Pasteur pipette and transferred to a 35 mm dish. The cells were disaggregated and incubated in complete medium.

Determination of Population Doublings. With mass cultures, population doublings were determined from the number of cells plated at each passage and the number of cells obtained at confluency. The total population doublings for the isolated clonal strains were estimated by (i) calculation of the number of doublings between colony isolation and growth to mass culture, assuming that each colony arose from a single cell, and (ii) addition of this value to the population doubling level achieved by the original treated culture, prior to plating for colony isolation.

Table 1. Cloning efficiency* in soft agar of normal and transformed cell lines of Syrian hamster embryo cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cells</th>
<th>Cloning efficiency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrian hamster embryo</td>
<td>10^6</td>
<td>0^*</td>
</tr>
<tr>
<td>BP6†</td>
<td>10^2</td>
<td>41 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>75 ± 6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>80 ± 15</td>
</tr>
<tr>
<td>BP6T†</td>
<td>10^2</td>
<td>96 ± 4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>BP6 + 10^6 hamster embryo cells‡</td>
<td>10</td>
<td>83 ± 8</td>
</tr>
</tbody>
</table>

* Number of colonies of 25 or more cells after 14 days of incubation in complete growth medium supplemented with 0.1% bovine serum albumin and 0.3% agar per number of cells suspended ×100%. The cloning efficiencies were calculated from the average of 3 to 10 determinations (for the low cell number experiment) at each cell density.
† No colonies were observed in 700 assays of 10^6 cells per assay. The cells were assayed from passage 3-20.
‡ Tumorigenic cell lines established from Syrian hamster embryo cells after exposure to BzP.
§ 10^6 normal hamster cells were plated with 10 BP6 cells.

Table 2. Summary of spontaneous transformation of Syrian hamster embryo cells

<table>
<thead>
<tr>
<th>No. of cultures assayed</th>
<th>Passage</th>
<th>Altered morphology*</th>
<th>Enhanced fibrinolytic activity†, %</th>
<th>Growth in soft agar‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>3–10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>15–20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4*</td>
<td>15–25</td>
<td>0</td>
<td>35–80</td>
<td>0</td>
</tr>
<tr>
<td>7†</td>
<td>30–40</td>
<td>0</td>
<td>~90</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>37</td>
<td>0</td>
<td>~90</td>
<td>3</td>
</tr>
</tbody>
</table>

* Percent of colonies judged morphologically transformed.
† Percent of colonies yielding a clear zone of lysis in the opalin fibrin/agarose overlay.
‡ Number of colonies comprising 25 or more cells formed in complete medium supplemented with 0.1% bovine serum albumin and 0.3% agar after 28 days of incubation per total number of cells (10^6) tested at various passages for growth in agar.
§ Cultures were characterized by a period of decreased growth rate and cloning efficiency followed by increased growth and cloning efficiency. At the time of increased growth, colonies with enhanced fibrinolytic activity were detected.
∥ Three mass cultures and four isolated colonies from the mass cultures were assayed.

RESULTS

Spontaneous Transformation of Syrian Hamster Embryo Cells. With the conditions employed in our laboratory, the hamster cells were grown in vitro routinely for 20 passages, approximately 80 population doublings. Colonies from control (dimethylsulfoxide treated) cultures remained morphologically normal and unable to grow in soft agar. Most control cultures senesced at or before passage 20 and failed to display colonies with enhanced fibrinolytic activity (Table 2). Approximately 1 of every 10 control cultures did not senesce, however, and after a period of decreased growth rate and cloning efficiency evolved at approximately passage 15–20 as a continuous cell line. Enhanced fibrinolytic activity of such cultures was then observed (13), although the increase in activity was only about 2-fold that of early passage cultures. These cell lines were not morphologically transformed. Colonies were isolated from control cultures at passage six (PTP-3 after dimethylsulfoxide treatment); however, the cells from over 20 colonies senesced after only one to four passages. At passage six no colonies with enhanced fibrinolytic activity were observed, so only colonies without detectable activity were isolated. Additionally, from the cultures that spontaneously evolved as established cell lines, six colonies with elevated fibrinolytic activity at PTP-15 were isolated; from these six, four cell lines were established and subcultured for at least 10 additional passages.

In order to test untreated cultures for cells capable of growth in soft agar, at least 10^6 cells from each of four groups of cultures were plated in the semisolid medium; the following cultures were assayed: (i) over 50 control mass cultures were grown until they senesced at approximately passage 20; cells from these cultures at early or late passages failed to grow in agar. (ii) Cells from four cultures which spontaneously evolved as cell lines were tested for growth in agar at passages 30–40 (after 150–200 population doublings), and no colonies in agar were observed. (iii) Four colonies, which were isolated from control cultures at PTP-15, when grown for an additional 15 passages after isolation (an estimated 173 total population doublings), still failed to grow in soft agar. (iv) Only cells from one control mass culture at passage 37 (185 population doublings) grew in agar at a frequency of three colonies per 10^6 cells. Therefore,
spontaneous transformation of Syrian hamster embryo fibroblasts to the ability to grow in soft agar is very rare, and can only occur after many population doublings.

**BzP Induced Transformation of Syrian Hamster Embryo Cells.** Treatment of the hamster cells with BzP induced morphological transformation, enhanced fibrinolytic activity, growth in soft agar, and tumorigenicity. The temporal acquisition of the in vitro phenotypes of a BzP-treated culture is shown in Table 3.

Both morphological transformation and enhanced fibrinolytic activity were observed within 2 weeks after carcinogen exposure; however, morphological transformation was observed before enhanced fibrinolytic activity (13). Anchorage independent growth was a later phenotypic change, not detected until 6–15 weeks (32–75 population doublings) after carcinogen treatment.

Between PTP-7 and PTP-12, overgrowth of the treated cultures by morphologically transformed cells was initiated; after approximately 2–4 additional passages, this cell type became predominant in the culture. These cells also possessed enhanced fibrinolytic activity. The development of the potential to grow in soft agar, however, did not appear to be related to this morphological alteration of the culture, because the appearance of cells capable of anchorage-independent growth varied from three passages before to six passages after the morphological change (data not shown).

In contrast to untreated cultures, cells treated with BzP gained the ability to grow in soft agar at a high frequency. Only one out of four treated cultures senesced. More importantly, cells from all the treated cultures that did not senesce developed the ability to grow in agar; this transformation was not detected until PTP-6–PTP-15 (32–75 population doublings after treatment).

The initial efficiency was one to three colonies in agar per 10⁶ cells, and the frequency of colony formation increased slightly in later passages, as shown in Table 3. Furthermore, the size of the colonies formed in agar increased with passaging. At PTP-9, the colonies in agar consisted of 25–50 cells after 14 days of growth, and they did not increase in size with additional incubation. The cells from cultures tested at later passages formed larger colonies after 14 days in agar, which continued to increase in size, up to several hundred cells per colony, upon further incubation.

**Tumorigenicity Studies.** Over 50 animals were injected with 2–4 million 10⁶ early passage untreated hamster cells, and 10 animals were injected with 10⁷ cells after 18 passages in vitro; no tumors were observed (15). Cells from four spontaneously established Syrian hamster embryo cell lines were injected into hamsters at 2 × 10⁶ cells per animal. Three of these cell lines, after 30–40 passages in vitro, failed to produce tumors in vivo. The fourth culture, cells of which formed colonies in agar at passage 37, produced tumors in three of six animals injected with 2 × 10⁶ cells at passage 40. An earlier passage of this line (passage 21) did not produce tumors.

Eight cultures treated with 1-10 µg/ml BzP for 24 hr failed to produce tumors when 2 × 10⁶ cells were injected at PTP-3–PTP-5. Six of these cultures transformed into cell lines capable of growth in soft agar. When these transformed cell lines were injected after their acquisition of anchorage independent growth, all the lines produced fibrosarcomas in 100% of the animals injected with 2 × 10⁶ cells. For example, the culture in Table 3 produced tumors when tested at PTP-13.

**Selection or Progression?** Two explanations may be offered for the delay in the appearance of cells capable of growth in agar. One explanation is that fully transformed cells capable of growth in semisolid agar were present in early passages, but at a level below the sensitivity of the soft agar assay, which is approximately 1 in 10⁶ to 10⁷ cells. If these cells had a selective growth advantage, they would be detected in the cultures at later passages. A second explanation is that neoplastic transformation in vitro is not a one-step process but a progressive process involving qualitatively different stages. To distinguish between these two possibilities, different colonies were isolated at an early passage after treatment. Following further culturing in vitro, each of these isolated cell lines was then examined for the presence of cells capable of anchorage-independent growth. At the time the initial isolations were performed, no colonies were detected in soft agar when 10⁶ cells from the mass culture were tested. In fact, the mass culture was tested at every passage after treatment, and no colonies were detected in agar until PTP-9 (46 population doublings after treatment, Table 3). These results clearly indicate that in early passages, the fraction of the population expressing the anchorage-independent phenotype had to have been exceedingly small. Therefore, if the transformation process had been completed at early PTPs and fully transformed cells were present, the probability of randomly isolating from the fibrin/agarose overlay a colony capable of growth in agar would have to be very low.

Nevertheless, a fraction of the population may have existed that had the potential for anchorage-independent growth, though the expression of this phenotypic characteristic was absent in early passages. For this reason, we searched for cells in the population at PTP-3 (13 population doublings after treatment) that would possess such potential, because we assumed that this period allowed sufficient time to establish any carcinogen-induced changes. As shown in Table 4, we were able, with a high frequency, to isolate colonies with the potential of expressing the anchorage-independent phenotype after additional culturing in vitro.

At PTP-3, nine colonies which possessed enhanced fibrinolytic activity were isolated from this culture. From these nine
attempted isolations, seven lines were established and subcultured for greater than 20 passages. Eventually, all of these lines developed the ability to grow in agar. When these seven isolated colonies were tested for growth in soft agar at 55 population doublings after BzP treatment, five cultures possessed cells capable of forming colonies in soft agar at a frequency of 5—100 colonies per 10⁶ cells. Cells from the other two cultures did not grow in agar until they reached a total of 75 population doublings. Additionally, fifteen colonies lacking detectable fibrinolytic activity were isolated; all of these isolates senesced in a similar manner as did the colonies from untreated cultures.

The theoretical fraction of the population at PTP-3 having the potential to grow in soft agar upon further growth can be calculated as follows (Table 4): 17.5% of the colonies from the treated mass culture possessed enhanced fibrinolytic activity at PTP-3. Of the nine isolates representative of such colonies, at least five developed the ability to grow in soft agar at 55 population doublings after treatment. Therefore, at least 9.7% of the total population theoretically had the potential to grow in agar after further growth in mass culture. Had these cells the full capability at PTP-3, then the theoretical number of colonies that would have been observed growing in soft agar at this early passage is 9.7 × 10⁶ per 10⁶ cells tested. Since, however, the observed number of colonies in agar depends also on the cloning efficiency in agar of these cells, a correction factor for this parameter was determined in the following manner. The colonies growing in agar, which were formed by the fibrinolytically active cells originally isolated at PTP-3 and grew in soft agar at 55 population doublings post-treatment, were isolated, grown to a sufficient number, and assayed for growth in agar. Five agar colonies were isolated and reassayed; the cloning efficiency of these cells was found to average 10 ± 5%. After correcting for this 10% plating efficiency, we determined that the theoretical number of cells that could grow in soft agar at PTP-3 should be 9.7 × 10⁶ per 10⁶ cells, a frequency which is easily detected by our methods (Table 1). In fact, when 10⁶ cells from the original cultures were assayed at PTP-3 for growth in agar, no colony was observed (Table 3). Therefore, we conclude that cells capable of anchorage-independent growth were not present at this stage in the transformation process, but, rather, developed only after additional growth in vitro.

DISCUSSION

In this communication, we have examined whether the cellular changes involved in neoplastic transformation in vitro can be described either by a simple, one-step process, or by a multistep progressive process through qualitatively different stages. The pattern of temporal acquisition of phenotypic alterations of Syrian hamster embryo cells after treatment with BzP is shown in Table 3. Morphological alterations were the earliest detectable changes, occurring within 1 week after carcinogen treatment (19), while enhanced fibrinolytic activity required a slightly longer time period of 2—3 weeks before expression. The development of cells with the capability of anchorage-independent growth required a significantly longer (6—15 weeks) period of in vitro growth. In fact, the latter phenotype developed in both mass culture (Table 3) and isolated colonies after approximately the same number of population doublings, 46—55. Additional studies on the expression of tumorigenicity are required, although our initial results indicate that it is delayed for a period of time similar to that for growth in agar.

In summary, 32—75 population doublings after carcinogen treatment were required for the development of anchorage-independent growth, a result which we cannot explain on the basis of selection for a small number of fully transformed cells within the population.

There are three possible reasons for this requirement of a long period before expression: (i) The phenotypic alteration represents merely a spontaneous transformation during in vitro culture and is not due to the carcinogen treatment. Under the present conditions of culture, only 1 of 10 untreated cultures developed into established cell lines and only one of five such cell lines spontaneously gained the ability to grow in agar, and this required 175 population doublings. It is improbable, therefore, that cells transformed to grow in agar at 40 population doublings after BzP treatment are the result of spontaneous transformation; rather, these cells are carcinogen-induced transformants. (ii) The delay in the expression of anchorage independence is due to a long period of phenotypic expression of one single event such as a somatic mutation, at the onset. This possibility cannot be completely excluded; however, a period of 30 to 50 population doublings is much longer than that required for the expression of most somatic mutations (16—18). In fact, somatic mutations at two genetic loci (hypoxanthine phosphoribosyltransferase locus and Na⁺/K⁺ ATPase locus) were assayed concurrently by using cells from these cultures. The expression time of these mutations was 6—8 population doublings after treatment (19). In addition, a fraction of the population—i.e., the fibrinolytically active colonies—was found to have a greater propensity to develop the ability to grow in agar. The enhancement of fibrinolytic activity could represent a developmental step preceding anchorage independence. For these reasons, we feel that the third explanation is most probable. (iii) This delay of expression indicates the requirement of secondary changes before the potential can be fully developed and manifested.

The explanation that BzP-induced transformation in vitro is not a single-step process but a multistep, progressive phenomenon, suggests a parallel between in vitro and in vivo carcinogenesis, and is in accord with the concept advanced by
Mondal et al. (20) of a two-stage in vitro carcinogenesis of mouse cells. Kakunaga and Kamahora (21) and Kuroki and Sato (22) have also suggested a progressive development of in vitro chemical carcinogenesis of Syrian hamster embryo cells. Recently, we have reviewed other plausible evidence in support of the progressive nature of in vitro transformation (23). Notably, the experiments of Laerum and Rajewsky (24) strongly support the concept of the progressive nature of in vitro carcinogenesis and the parallelism of in vitro and in vivo carcinogenesis. Their studies indicate that the time requirement for progression from initiation to the tumorigenic state was nearly equal for both in vitro and in vivo carcinogenesis of fetal rat brain cells. The results of our studies and those of the above mentioned investigators justify the study of oncogenesis in cell culture as a model for neoplastic transformation in vivo.

Our attempt to define and detect preneoplastic cells in the transformation process are of paramount importance. In this regard, understanding the relevance and interrelationships of phenotypic changes may be very useful. Our studies demonstrate an independent temporal acquisition of the phenotypic transformations of morphology, enhanced fibrinolytic activity, and the ability to grow in soft agar. Kakunaga and Kamahora (11) have reported morphologically normal cells that grow in soft agar. We have obtained similar cell lines and demonstrated that they possess enhanced fibrinolytic activity (unpublished data). These results suggest an independent development of each of these phenotypes associated with malignancy. This is consistent with the independent progression of different characters of a tumor in vivo.

Each of the in vitro correlates of neoplasia we have examined may be a spurious consequence of the transformation process and may have no necessary function in the maintenance of the malignant state. However, growth in soft agar appears to correlate well with tumorigenicity (11), which suggests that the changes required for anchorage independence are important for tumor formation. Our results demonstrate that morphological transformation and enhanced fibrinolytic activity are not sufficient for tumorigenicity in Syrian hamster embryo cells, because late passage cells in untreated cultures possess enhanced fibrinolytic activity, yet they lack tumorigenicity and do not readily transform spontaneously into soft agar-positive cells. However, when fibrinolysis-positive colonies are isolated from carcinogen-treated cultures and continuously cultured, over 50% give rise to cells capable of growth in soft agar. This may suggest a role for fibrinolysis in neoplasia, although the possibility that enhanced fibrinolytic activity is merely a consequence of the in vitro transformation process cannot be excluded (13).

In summary, we have presented evidence that neoplastic transformation in vitro is a progressive event analogous to in vivo carcinogenesis. This realization of the complex nature of neoplastic transformation in vitro should caution against the simplistic approach of applying "short-term" in vitro assays for quantitative risk assessment of suspected carcinogens.

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