Repopulation of Denuded Tracheal Grafts with Normal, Preneoplastic, and Neoplastic Epithelial Cell Populations

Margaret Terzaghi,² Paul Nettesheim, and Mary Lou Williams

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

ABSTRACT

The purpose of these experiments was to study the in vivo growth characteristics of nonmalignant and malignant epithelial cell cultures maintained in vitro for various lengths of time. All cell cultures tested originated from normal or carcinogen-exposed tracheal epithelium of Fischer 344 rats. The cultures were inoculated into isolated tracheas, which were then grafted to isogenic recipients. At least 10⁶ epithelial cells were required to reestablish a complete epithelial lining in denuded tracheal grafts, whether normal or tumorigenic cell cultures were used. Inoculation of denuded tracheas with 1-week-old primary cultures of normal tracheal epithelium resulted in the reestablishment of a nearly normal mucociliary tracheal lining within 1 to 2 weeks. Injection of primary epithelial cultures older than 1 week failed to reestablish an epithelial lining; instead, the tracheal lumen became obliterated with connective tissue. Inoculation with preneoplastic epithelial cell lines resulted in the establishment of a well-organized, keratinizing squamous epithelium, which remained stable for at least 6 weeks if the recipient was immunosuppressed, but which was rejected at 4 weeks in immunocompetent hosts. Inoculation with a neoplastic cell line resulted in the establishment of a well-organized squamous epithelium for 3 to 4 weeks and the development of disorganization, exophytic growth, and invasion at 4 to 6 weeks. This occurred only in immunosuppressed recipients. In immunocompetent hosts the epithelial lining was completely rejected within 2 weeks. When cells from a highly malignant squamous cell carcinoma line were inoculated into tracheas with an intact epithelial lining, only a few isolated nests of malignant cells were observed at 6 weeks. Repopulation of denuded tracheas with cells from the same tumor line resulted in the establishment of an atypical squamous lining at 1 week. At 2 weeks invasion was widespread, resulting in destruction of the tracheal grafts shortly thereafter.

The epithelial morphologies observed in these studies are highly reminiscent of various preneoplastic and neoplastic "lesions" induced in tracheas of rats by direct action of chemical carcinogens. Our experiments show that this "in vivo culture system" is well suited to the study of growth and differentiation characteristics of carcinogen-altered or preneoplastic epithelial cell populations.

INTRODUCTION

The development of neoplasia is presumed to occur as a multistage process in which "initiated" cells gradually acquire neoplastic characteristics (6). Sequential cell and tissue changes have been observed in many tissues, notably epithelial organs, following exposure to carcinogens (e.g., see Refs. 1, 5, 7, 9, 21, and 23). However, for a number of technical reasons it is difficult to obtain conclusive evidence for this developmental hypothesis of carcinogenesis, since it is generally not feasible in vivo to take repeated samples from a "lesion" presumed to be composed of preneoplastic cells. Without the possibility of repeated sampling from the same cell population, precursor-progeny relationships between sequentially occurring lesions cannot be established unequivocally. It appears, therefore, that in some respects cell culture systems are better suited to the study of problems of "neoplastic progression," since pure cell clones can be obtained and the same cell population can be studied and analyzed over extended periods. However, the in vitro approach has other problems and limitations. The differentiated state of epithelial cell populations is altered in existing culture systems (2). Artifacts may develop because of repeated passage and rapid continuous proliferation, which in turn can result in artificial selection of cell subpopulations. In addition interactions between carcinogen-altered cells and host-specific factors (22, 24), epithelial-mesenchymal interactions (3, 11, 18, 19), and interactions between preneoplastic and adjacent normal cells (25, 26), which presumably affect neoplastic progression in vivo (3, 18, 20, 22), do not occur in cell cultures in vitro. The morphological markers thought to be characteristic of different stages of the evolution of neoplastic disease in certain epithelial systems (1, 5, 7, 9, 21, 23) have not been recognized in vitro. It clearly would be advantageous to have an experimental system that incorporates desirable features of both in vitro and in vivo systems.

We have previously reported the use of tracheal grafts as an experimental tool in the study of the development of carcinogen-induced lesions in a preselected segment of respiratory tract mucosa (8, 9). Nair and Crocker (17) suggested that such tracheal grafts might be useful in studies of epithelial-mesenchymal interactions if the origi-
nal epithelial lining could be destroyed and variously pre-
treated epithelial cells could be implanted.

In this report we describe the transplantation of rat tracheas denuded of their epithelium to allow repopulation by normal, preneoplastic, and neoplastic cells. In this manner epithelial cell lines that were originally isolated from tracheas exposed to carcinogen for various in vitro studies (9, 14, 15) were cultured in vivo in an environment approaching that of the organ of origin. Because multiple aliquots from the same in vitro cell population can be inoculated into denuded tracheal grafts, sequential morphological studies of cells derived from the same original population could be carried out. Also described are the interactions of the preneoplastic and neoplastic cell lines with normal tracheal epithelium and with host immune mechanisms. We believe that this in vivo culture system will facilitate the investigation of many crucial problems in neoplastic development.

MATERIALS AND METHODS

Animals. Female Fischer 344 rats raised and maintained under specific-pathogen-free conditions were used. For the induction of severe immunosuppression, 4- to 5-week-old rats were thymectomized according to standard procedures (16). After 3 weeks they were exposed to 600 rads whole-body X-irradiation; this was followed by a second X-ray exposure of 400 rads 2 weeks later. An i.p. injection of 1 to 2 × 10⁶ bone marrow cells was given immediately after the second X-ray dose to assure survival. Animals were used as recipients of tracheal grafts within 24 hr after their last X-ray exposure.

Tracheal Grafts. Tracheas were aseptically removed from anesthetized 8- to 12-week-old rats; they were transplanted s.c. to the subscapular region of isogenic recipients, 2 tracheas/recipient. The methods used have been previously described (8). In the present study tracheas were used as "receptacles" for various epithelial cell cultures to study their in vivo growth behavior. Three different types of tracheas were used for this purpose: tracheas with normal epithelial lining; tracheas with part of the epithelial lining removed; and tracheas with all of the epithelial lining removed. Partial removal of the epithelium was achieved by "scraping" the side opposite the pars membranacea with a scraping needle fashioned from a 16-gauge needle. Approximately one-half of the tracheal lining was denuded of epithelium. For complete removal of epithelium from tracheas, the following procedure was adopted after a number of other methods were found unsatisfactory. As much epithelium as possible was removed with the scraping needle, and then the tracheas were maintained at 37° for 6 to 8 days in serum-free Ham's F-12 or Waymouth's medium. In 90% of the cases, this procedure resulted in epithelium-free tracheas with viable cartilage and stroma. The effectiveness of the method was tested by transplanting tracheas so treated and by culturing small tracheal pieces in vitro under conditions similar to those previously described for the establishment of outgrowths (9, 14). If all the epithelium had been removed, tracheas histologically examined 2 weeks after transplantation were totally devoid of epithelium and filled with young connective tissue. The cartilage was viable as indicated by normal staining with hematoxylin-eosin and by the presence of cells in the lacunae. The in vitro outgrowth procedure yielded viable fibroblasts, indicating that at least some of the mesenchymal components had survived the prolonged incubation in serum-free medium. Other methods with the use of enzymatic removal of epithelium and X-irradiation in combination with various incubation procedures were not satisfactory.

Epithelial Cell Cultures. Three types of epithelial cell cultures were used for tracheal repopulation studies: primary tracheal cell cultures and 2 established tracheal cell lines, one neoplastic (BP₁) and the other (1000 M) preneoplastic at passage 7 and neoplastic at passage 20. Primary epithelial cell cultures were established as follows. Tracheas freshly removed from donor rats were filled with 0.25% pronase (protease type IV; Sigma Chemical Co., St. Louis, Mo.) in Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.) and were incubated overnight at 4° and for an additional 15 min at 37°. Cells were removed by scraping with the scraping needle and by repeatedly flushing the lumen with medium. Cells obtained from 3 to 5 donors were pooled. The resulting cell suspension was washed 3 times by sequential centrifugation, and the cell pellet was resuspended in Ham's F-12 medium containing 10% fetal calf serum. Cells were seeded at a density of 5 × 10⁴ cells/60-mm culture dish in Ham's F-12 plus 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml), insulin (0.1 μg/ml), and hydrocortisone (0.1 μg/ml). Primary cultures were used for tracheal repopulation studies within 1 week of seeding in vitro. Microscopic studies of fixed and stained cultures showed that roughly 10% of the attached cells were fibroblasts. Two established cell lines were used for tracheal repopulation studies. One line, designated BP₁, was a malignant epithelial cell line derived from a tracheal squamous cell carcinoma that had been induced in vivo with benzo(a)pyrene (13). The fourth in vitro passage was used in our studies. This cell line regularly produced squamous cell carcinomas upon i.m. inoculation of 10⁶ cells into isogenic rats, with latency periods (i.e., time to palpable nodule) of 10 to 12 days. The other epithelial cell line, designated 1000 M, originated from a dimethylbenz(a)anthracene-exposed trachea by means of the explant outgrowth method previously described (14, 15). Once the cell line was established, cells were passaged every week at a split ratio of 1:10. The 7th and 20th passages were used in this study. Inoculation of 10⁶ cells from either passage into immunocompetent isogenic hosts did not produce any tumors. Inoculation of 10⁶ cells from passage 7 into immunosuppressed recipients resulted in the formation of keratinic cysts (first palpable after 30 weeks) in 1 of 4 cases. Inoculation of 10⁶ cells from passage 20 into immunosuppressed hosts resulted in the development of squamous cell carcinomas within 30 to 40 days (i.e., time to palpable nodule) in 4 of 4 cases.

Inoculation of Tracheas with Cell Cultures. Cell cultures were dissociated by incubating them with 0.2% trypsin and 0.25% EDTA in Ca²⁺-Mg²⁺-free Hanks' solution plus 5% chicken serum. The resulting cell suspensions were centrifuged and then resuspended in fresh medium so that the desired cell inoculum was contained in 50 μl of culture medium. Aliquots (50 μl) were inoculated into freshly pre-
pared tracheas closed off at the bifurcated end. The laryngeal end was then tied with a suture, and the tracheas containing the cell inocula were transplanted within 1 hr into the subscapular region of suitably prepared recipients.

RESULTS

Repopulation Studies with Normal Tracheal Cell Cultures. The aim of these studies was to determine whether inoculation with cells from 1- to 6-day-old primary tracheal epithelial cultures can establish a normal mucociliary epithelium in tracheal grafts denuded of their own epithelium. This study also serves as a control for other experiments in which the in vivo growth behavior of preneoplastic and neoplastic tracheal cell cultures are similarly studied. Tracheas denuded of their epithelium were inoculated with $5 \times 10^6$ viable cells as determined by means of trypan blue dye exclusion from primary cultures of tracheal epithelial cells. Such cultures consist of approximately 90% epithelial cells, similar in appearance to the poorly differentiated cells described in outgrowths of tracheal explants (10). Denuded tracheas without cell inocula served as controls. When these control tracheas were histologically examined 1 to 6 weeks later, it was found that their lumina were completely obliterated by young granulation tissue. In only about 10% of the cases did we find remnants of epithelium forming small nests or irregular channels; the rest of the former tracheal lumen was filled with connective tissue (Fig. 1). In contrast denuded tracheas inoculated with $5 \times 10^6$ cells from tracheal cell cultures always showed a patent lumen of normal width, lined by epithelium. At 1 week cross-sections of tracheas showed approximately one-half of the surface lined by low cuboidal and half by mucociliary epithelium. At 2 weeks all tracheas were completely lined by hyperplastic mucociliary epithelium (Fig. 2). Between 3 and 6 weeks, a quiescent normal tracheal epithelium was found in all tracheas. Only occasional small patches of low cuboidal or atrophic epithelium remained. Alcian blue-periodic acid-Schiff-stained sections showed mucus-containing cells, and the tracheal lumen was filled with mucus.

A cell dose study with $10^6$ to $10^7$ viable cells inoculated per trachea showed that $10^6$ was the minimum number of cells required to reestablish an epithelial lining throughout the entire tracheal graft. Attempts to repopulate tracheas with up to $10^8$ viable cells from primary cell cultures older than 6 days were unsuccessful. This observation suggests that, although the cells were “viable” (as determined by dye exclusion), with increased time in vitro the number of progenitor cells that are capable of proliferating and differentiating in vivo rapidly declines.

Repopulation Studies with Preneoplastic and Neoplastic Tracheal Cell Cultures. Denuded tracheas were inoculated with preneoplastic (1000 M, passage 7) and neoplastic (1000 M, passage 20) cell cultures to determine whether significant differences could be observed between the repopulation patterns of the 2 cell lines. As described in “Materials and Methods,” at passage 7 the line 1000 M yielded only a low incidence of keratonic cysts when inoculated i.m. into immunosuppressed rats. The same cell line at passage 20 regularly produced invasive squamous cell carcinomas upon i.m. injection into immunosuppressed recipients. Passage 7 was, therefore, considered to be preneoplastic. Denuded tracheas were inoculated with $5 \times 10^6$ cells of either passage 7 or 20 and were transplanted into immunosuppressed recipients. Tracheal transplants were sampled 1, 2, 3, 4, and 6 weeks later. During the first 4 weeks after cell inoculation and transplantation, the tracheas repopulated with the cells from the 2 different passages were similar. The tracheal surfaces were uniformly lined by a layer of keratinizing squamous epithelium 3 to 6 cells thick. Only subtle differences were noted between the 2 sets of tracheas. The epithelial linings established with cells from passage 20 (Fig. 3a) showed more mitotic figures and more cellular atypia (variation in size and shape of nuclei, coarser chromatin, more prominent nucleoli) and were generally thicker and more heavily keratinizing than those established with cells from passage 7 (Fig. 3b). Occasional small epithelial cell nests were seen in the submucosa of denuded tracheas after inoculation with cells from either passage. Since they did not noticeably expand during the entire observation period, they were interpreted as being cells trapped in the submucosa during the early regenerative process. At 6 weeks marked differences between the 2 types of epithelial linings became noticeable. Those established with passage 7 cells still formed a thick, keratinizing squamous epithelium with a well-delineated epithelial-mesenchymal border. In contrast the lining made up of cells from passage 20 showed signs of extensive endophytic and exophytic growth. Invasion into the submucosa was clear in all tracheal cross-sections (Fig. 4). The cells showed severe cellular atypia and parakeratosis, which was not noted at earlier time points, was widespread (Fig. 4a). At 10 weeks the neoplastic cells (1000 M, passage 20) had already grown far beyond the boundaries of the tracheal transplant into host tissues.

The same study was also performed in immunocompetent hosts. We found that the early repopulation (1 week) of tracheas with the 2 different passages from the 1000 M cell line was similar to that observed in immunosuppressed hosts. However, 3 weeks after inoculation with passage 7 cells and 2 weeks after inoculation with passage 20 cells, massive infiltration of the tracheal submucosa and the newly established tracheal lining with mononuclear cells was observed (Fig. 5a). In the subsequent 1 to 2 weeks, the entire epithelial surface sloughed off in both sets of tracheas (Fig. 5b). The apparent rejection process clearly seemed to occur more rapidly in tracheas repopulated with passage 20 cells. That this rejection reaction was directed specifically against the epithelial cells of repopulated tracheas was evident from studies with partially denuded tracheas. Using passage 7 cells we observed that, during the 4 weeks in which the study was conducted, passage 7 cells apparently remained confined to that area of mucosa stripped of the native epithelium, respecting the boundaries of the remaining epithelial lining. Severe mononuclear cell infiltration occurred mostly in that part of the tracheal mucosa lined by 1000 M cells (Fig. 6).

Repopulation Studies with a Highly Malignant Squamous Cell Carcinoma. Experiments were carried out with cells from a highly invasive squamous cell carcinoma.
Intratracheal Growth in Vivo of Epithelial Cells

(BP), induced in vivo by benzo(a)pyrene. The cell dose study conducted with these tumor cells (dose range, $10^3$ to $5 \times 10^6$ cells) showed that at least $10^4$ cells are required to repopulate completely denuded tracheas. The time course study conducted over 4 weeks following inoculation of $10^4$ or more BP cells into denuded tracheas showed that the tumor cells grew for the first 1 to 2 weeks as a solid epithelial sheet in some areas of the trachea (Fig. 7) and as an invasive tumor in other areas. At 3 and 4 weeks, the tracheal lumen was filled with tumor masses, and much of the tracheal graft had been destroyed by invading neoplastic cells. Tracheas inoculated with $10^6$ cells were obliterated with fibrous tissue, and no signs of tumor development or of surviving epithelial cells were found as late as 4 weeks after cell inoculation.

Interaction of Normal Tracheal Epithelium and Squamous Carcinoma Cells. For determination of whether tumor cells implant and grow on the surface of normal tracheal epithelium, tracheas with an intact epithelial lining were inoculated with $5 \times 10^5$ BP cells and transplanted to normal recipients. Two tracheas each were sampled at 2, 3, and 4 weeks after grafting. Each trachea was cut into 2-mm-thick cross-sections. From each cross-section 4 histological step sections, 100-μm apart, were obtained. In 3 of the 6 tracheas examined, there were microscopic tumor cell nests (0 and 1 at 2 weeks, 1 and 2 at 3 weeks, 0 and 2 at 4 weeks). Initially, they were located on the surface of and within the tracheal epithelium (Fig. 8). By 4 weeks these cell nests, although still relatively small, had begun to grow exophytically and endophytically, showing early submucosal invasion. Initially, the growth seemed to occur mostly in a vertical, rather than in a horizontal, direction relative to the basement membrane. At later times the tumor cells destroyed and replaced the normal epithelial lining.

DISCUSSION

Our studies illustrate that denuded tracheal grafts support the growth and differentiation of normal, preneoplastic, and neoplastic respiratory epithelium. The method used to remove and destroy the original epithelial lining produces a completely denuded and apparently viable graft in most cases. That the grafts remain viable at least in part is suggested by our observation of fibroblast outgrowth from denuded tracheal explants in vitro. However, we have to assume that host-derived mesenchymal elements invade the tracheal graft as it becomes vascularized. Our studies with heavily X-irradiated tracheas support this notion, since the grafts become readily established and populated with mesenchymal elements. Similar observations were reported by Nair and Crocker (17). If the denuded tracheas are not seeded with at least $10^4$ normal or neoplastic epithelial cells, their lumina become obliterated with connective tissue within 1 week. How the epithelium succeeds to control the proliferation of connective tissue is unknown. Somehow the inoculated normal as well as neoplastic epithelial cells succeed in achieving a balance between epithelium and mesenchyme that closely mimics the normal state.

The normal epithelial cells used in the repopulation studies were obtained from primary cultures of dissociated tracheal epithelium. These cultures are monolayers composed of mostly "primitive" epithelial cells, which to a large extent do not express the typical features of ciliated and mucous cells as described in tracheal outgrowth cultures (10). The tracheal repopulation studies suggest that some fraction of these dissociated cells had, up to 1 week after seeding in vitro, maintained their normal differentiative potential, which is reexpressed when they are allowed to grow in denuded tracheas. Older cultures had lost this potential, a finding that is similar to observations made by Dodson (4) in epidermal cell cultures. Whether any specific cell-cell interactions within the reconstituted trachea are operative in the epithelial cell differentiation during tracheal repopulation is presently unknown.

The repopulation studies with the preneoplastic and neoplastic phase of one of our cell lines (1000 M) demonstrated that, before expressing the malignant phenotype (i.e., invasiveness), neoplastic cells behave much like preneoplastic cells. The cells from both passages of the 1000 M cell line initially grew as well-organized squamous epithelium distinguished only by subtle cytological and histological differences. Only after 4 weeks did passage 20 cells begin to show marked cellular atypia. This was followed by exophytic growth and invasion of the tracheal stroma by this neoplastic population 6 weeks after cell inoculation. Similar observations were made on the tumor with cells from the BP carcinoma line, which is known to form highly malignant tumors. Initially, the cells grew, at least in some areas, as a multilayered epithelial sheet covering the surface provided to them. What trigger mechanism prompts cells in other areas to pile up and/or focally invade is not known. Our observation supports the concept, implicit in terms such as carcinoma in situ, that absence of invasion does not mean absence of the capacity to invade.

Another interesting observation is that the preneoplastic epithelium, unlike neoplastic epithelium, apparently does not readily displace normal epithelium. Studies in which the normal epithelium was only partially removed from parts of the trachea before preneoplastic cells were inoculated into the lumen suggest that the preneoplastic epithelium stayed within the confines of the scraped area and "respected" the boundaries of the normal epithelium. This also appeared to be the case for neoplastic cells for the first 1 to 2 weeks after cell inoculation. Only after the neoplastic cells had started to invade the submucosa massively was the architecture of the normal epithelial surface disrupted.

The studies in which normal tracheas, with an intact epithelium, were seeded with neoplastic cells (BP) indicated that the normal epithelium provides an effective, though not absolute, barrier against the implantation of these cells. In totally denuded tracheas inoculated with the same number of neoplastic cells, a large invasive tumor mass was observed within 2 weeks of transplantation. In tracheas with an intact epithelial lining, only a few isolated small tumor cell nests were observed 4 weeks after transplantation. The fact that these foci apparently grew relatively slowly suggests that the surrounding normal epithelium may have exerted some type of growth inhibition on the neoplastic cell nests. The observation that normal tracheal epithelium presents a partial barrier to tumor cell growth.

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 1978 American Association for Cancer Research.
implantation is similar to that reported by Weldon and Soloway (27) for the bladder. The apparent delay in expression of the malignant phenotype observed in our experiments may be similar to that in cases cited by Pierce and Fennel (20) in which the malignant phenotype was effectively controlled in certain environments.

Finally, the studies with the early and late passages of the 1000 M cell line clearly demonstrated an immunological rejection response in the tracheal grafts. This response was more rapid with the neoplastic cells from passage 20 than with preneoplastic cells from the early passage. We have previously shown that chemically induced tracheal carcinomas are antigenic (13). We have shown that the expression of antigenicity of such tumors can be enhanced by long-term in vitro maintenance of tumor lines (12). The present finding is particularly interesting because it suggests that the tumor-associated antigens described in our previous studies with respiratory tract carcinomas may already appear in the preneoplastic stages. In this regard similar observations have been made by Slemmer (25) in premalignant mouse mammary tumors. However, since the cell lines studied in the present experiments were grown in culture medium containing fetal calf serum, part of the immunological response could have been triggered by heterologous antigens.

REFERENCES

Intratracheal Growth in Vivo of Epithelial Cells
Repopulation of Denuded Tracheal Grafts with Normal, Preneoplastic, and Neoplastic Epithelial Cell Populations

Margaret Terzaghi, Paul Nettesheim and Mary Lou Williams


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/38/12/4546

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.