Differentiation of Malignant to Benign Cells

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SUMMARY

Rats bearing well-differentiated transplantable squamous cell carcinomas were given injections of thymidine-3H, and the tumors were examined at intervals of time by radioautography with light and electron microscopes. Two hr after injection, labeled cells were almost exclusively in the undifferentiated areas of the tumor, and 96 hr after injection many cells of the well-differentiated pearls were labeled. These data suggest that the growth of the tumor was dependent upon proliferation of undifferentiated cells and that the growth of the pearls was dependent upon incorporation of undifferentiated cells into the pearl. Labeled cells that migrated into the pearls became well differentiated, as determined by electron microscopic radioautography, and when transplanted into compatible hosts did not form tumors. Thus it would appear that the progeny of malignant stem cells can differentiate into postmitotic benign cells incapable of forming a tumor.

INTRODUCTION

Many tumors are inhomogeneous when examined by light microscopy. Squamous cell carcinomas have "pearls" of well-differentiated squamous cells surrounded by large masses of undifferentiated cancer cells. In a similar manner, osteosarcomas or chondrosarcomas may have foci of recognizable bone or cartilage, respectively, embedded in masses of highly malignant cells. The first and most widely accepted idea concerning the mechanism responsible for this heterogeneity was that there were variable degrees of dedifferentiation as a mechanism of carcinogenesis, the idea that many of the progeny of malignant stem cells of tumors in general might have a capacity for differentiation (19), accounting for the heterogeneity of cells present. This idea is supported by other recent studies (8, 15, 27).

In a further test of the concept that some of the progeny of malignant stem cells are able to differentiate, we labeled the undifferentiated cells of a transplantable squamous cell carcinoma of rats, followed their ability to differentiate, and then tested the biological behavior of the resulting differentiated squamous cells. The data obtained support the idea that many of the progeny of the malignant stem cells of this tumor differentiate into apparently benign postmitotic squamous cells.

MATERIALS AND METHODS

The tumor used in these experiments was obtained from Dr. Katharine Snell of NIH. The tumor grew slowly and did not metastasize. It developed near the lip following prolonged feeding of a carcinogen (9) to Irish rats. Explants of this tumor grew slowly in the s.c. space in the rats, and a large inoculum (about 0.25 ml of 50% suspension of tumor) was required to ensure 100% transplantability. Such grafts grew to a diameter of about 2 to 4 cm in 3 to 4 months, and they always destroyed their host, although a prolonged period was required. Microscopically, the tumor was well differentiated with numerous squamous pearls separated from each other by undifferentiated cancer cells with many mitoses (Fig. 1). In addition, there were large cells with clear cytoplasm and large red granules that often lay in clumps by themselves or in relationship to the pearls. These cells were considered to be a type of differentiation toward the dark cells of eccrine sweat glands. This tumor did not metastasize but caused death by cachexia with terminal infection.

Rather than clone this tumor, as was done with the teratocarcinoma (10), we labeled proliferating stem cells with tritium-labeled thymidine, and their ability to differentiate was followed by radioautography with light and electron microscopes. Tritium-labeled thymidine (specific activity, 6.7 Ci/mM) was acquired from New England Nuclear Corp., Boston, Mass. Each animal received 1 μCi of tritium-labeled thymidine/g of body weight. The animals were sacrificed at the times listed in Table 1, and tissues were processed for radioautography with the use of the techniques of Kopriwa and Leblond (11). In the initial experiments, 1 animal was examined at each time interval; in the confirmatory one, 3 additional animals were used per interval of time. All somatic tissues of the teratocarcinomas and that these somatic tissues were benign (21). This evidence was compatible with the idea that some of the progeny of malignant stem cells of tumors in general might have a capacity for differentiation (19), accounting for the heterogeneity of cells present. This idea is supported by other recent studies (8, 15, 27).

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specimens in an experiment were processed simultaneously, and each experiment was performed similarly under controlled conditions so comparisons could be made between groups. Cells with more than 6 grains were considered to be labeled.

Two animals were used for each interval of time for electron radioautography. They received 20 μCi of tritium-labeled thymidine/g of animal, and the tissues were processed with the use of a modification of the techniques of Rogers (24).

Since the classification of tumors as benign or malignant is a clinical one measuring the effect of tumor on host, we tested and compared the biological behavior of the undifferentiated clinical one measuring the effect of tumor on host, we tested and compared the biological behavior of the undifferentiated one employing the use of a modification of the techniques of Rogers (24).

Results

Radioautography with the Light Microscope. For the light radioautograms, representative sections of each tumor were studied with notation of all labeled nuclei and whether they were located in undifferentiated cancer or in squamous pearls. Tumors were not uniformly labeled, suggesting that DNA synthesis occurred in certain regions at certain times. The results of the initial and confirmatory experiments agreed closely and have been combined in Table 1.

Two hr after injection of tritium-labeled thymidine, many nuclei in the undifferentiated parts of the cancer were labeled (Fig. 2), but only 5 labeled nuclei were found in the 779 pearls examined. Three of these labeled nuclei were on the margin of the pearls and may have been nuclei of undifferentiated cells. Chart 1 is a histogram illustrating the number of grains per labeled nucleus in undifferentiated cancer cells 2 hr after the administration of the pulse label. Most labeled cells had in excess of 38 grains.

Evidence in Chart 1 and Figs. 3 and 4 indicates that there was a gradual increase in the number of labeled nuclei per pearl with time during the 4 days of the experiment. At 28 and 56 hr, labeled cells were near the margin of the pearls, but later they were randomly scattered in the pearls. A histogram (Chart 2) illustrating the number of grains per labeled nucleus of undifferentiated stem cells and differentiated squamous cells indicates 2 peaks, at 22 and 20 grains, respectively. Since the labeled nuclei in the pearls had about 50 or 25% of the grains that the labeled undifferentiated cells had at 2 hr, at least 1 cell division must have occurred before the undifferentiated cells migrated into the pearls (Chart 2) as in the normal situation. This observation is at variance with that of Frankfurt (6), who believes that labeled malignant cells about to differentiate do not undergo a mitosis. It was concluded from these data that cells in the pearls were not synthesizing DNA and that growth of the pearls depended upon incorporation of undifferentiated cancer cells into the pearls with subsequent differentiation. Radioautography with the electron microscope and transplantation of pearls were undertaken to confirm this idea.

Electron Microscopy. The undifferentiated areas of the tumor were characterized by a mosaic of cells with nuclei that were either round or oval with large but conventional-appearing nucleoli (Fig. 5). The plasma membrane lacked desmosomes, and there was little or no interdigitation of plasma membranes between adjacent cells. Mitochondria with dark-staining matrices were scattered throughout the cytoplasm. Ribosomes, both free and integrated, were the dominant granules of the cytoplasm, although a few profiles of thin and attenuated...
rough endoplasmic reticulum were present. Microtubules and filaments were rare. Occasional osmiophilic inclusions were present. A single Golgi complex was occasionally visualized, but the overall impression gained was that this was an undifferentiated cytoplasm (22, 30) characteristic of rapidly proliferating cells.

The pearls were usually separated from the undifferentiated stem cells of the carcinoma by a thin basement membrane. Small pearls had only 2 strata of undifferentiated cells, whereas large pearls had about 4 each with increased degrees of differentiation toward the center. The cells lining the basement membrane usually had relatively electron-lucent ground substance and, depending upon the size of the pearl, appear either crescent shaped (in small ones) or cuboidal (in large ones). In addition to mitochondria with relatively dark-staining matrices, the cytoplasm contained numerous microtubules and filaments and occasional membrane-bound inclusions. The plasma membrane contained desmosomes, which connected adjacent cell membranes. The desmosomes were always most numerous in the membranes adjacent to the next stratum of better-differentiated cells. These cells resembled those of basal cell carcinoma described by Zelickson (Fig. 6) (31). In the next stratum of cells, polyvesicles and free ribosomes were numerous, and many cisternal profiles of endoplasmic reticulum containing some electron-lucent material were present. Microtubules were commonly found, and tonofibrils inserting into desmosomes were numerous. A few membrane-bound inclusions and a rare keratohyaline granule were found in the cytoplasm of these cells. The next layer of cells closely resembled the stratum spinosum of normal squamous epithelium (Fig. 6). The nuclei of this stratum as well as others were usually flattened and somewhat crescent shaped, in conformity with the shape of the pearl. Desmosomes occurred frequently in cell membranes that were markedly folded with many microvilli. Polysomes were particularly numerous and were randomly distributed between bundles of tonofibrils. Desmosomes and tonofibrils were more numerous on the side of the cell near the center of the pearl. Small membrane-bound inclusions averaging about 200 μm were often found near the cell membrane and tonofibrils. These were osmiophilic and were also found in the next layer of differentiation. They resembled membrane-coating granules (17). In the next layer of cells, keratohyaline granules were present, ranging in size from very small to extremely large (Figs. 7 and 8). Differentiated organelles were scarce, and their numbers varied inversely with the amount of keratohyalin in the cells. Cell membranes were regular and had many desmosomes with attached tonofibrils. In the layer immediately adjacent to the keratotic center of the pearl, the nuclei and membranous organelles of the cells were lost, and the flattened cells were composed of dense bands of keratohyalin and cell membranes in parallel array.

In addition to squamous differentiation, cells were present in the pearls that bore a close resemblance to the dark cells of eccrine sweat glands (3). The cells were irregular in shape with oval nuclei and smoothly contoured plasma membranes. Microvilli were rarely found between dark cells and other cells of the pearls. The most prominent feature was the presence of numerous, intensely osmiophilic granules contained within smooth membranes. In addition, there were other vacuoles of varying size which contained a relatively electron-lucent material. Membranous whorls were also present among the secretion granules, but the origin of the membranous material was not evident from the micrographs. Cells resembling sebaceous cells were occasionally seen.

Radioautography with the Electron Microscope. Two hr after injection of tritium-labeled thymidine, labeled nuclei were found in the undifferentiated area of the tumors (Fig. 5). The labeled cells, characterized by a marked paucity of endoplasmic reticulum and Golgi complexes and by the presence of a large number of polysomes, were considered to be undifferentiated stem cells.

Many labeled nuclei were present in the pearls 56 hr after a pulse dose of tritium-labeled thymidine (Figs. 6 to 8). Although it is not the purpose of this paper to describe the ultrastructure in great detail, it is readily apparent from Figs. 6 to 8 that the pearl represents a caricature of epidermogenesis and that the labeled cells were well differentiated.

Transplantation Experiments. Of 82 transplants of undifferentiated cancer, 27 developed into tumors by the end of the 7th month after transplantation. The largest of these was 2.5 cm in diameter; the smallest was approximately 2 mm in diameter. Six of the tumors had attained a diameter of 2 cm by the 4th month after transplantation. In our experience, each of these tumors would have destroyed its host. Microscopically, they closely resembled the control tumors, although there was considerable variation in the incidence of dark cells.

Of 78 transplanted pearls, none developed into a squamous cell carcinoma. Care was taken to ensure that the transplanted pearls contained less than 10% nonviable keratin and were composed of viable squamous cells, as illustrated in Figs. 6 to 8. It was concluded from this experiment that the well-differentiated cells of the squamous pearls are incapable of synthesizing DNA, are postmitotic, and cannot form a tumor.

DISCUSSION

The data obtained 2 hr after injection of a pulse dose of tritium-labeled thymidine indicate that the squamous cells of the pearls were incapable of synthesizing DNA and that the growth of the tumor was dependent upon the proliferation of undifferentiated stem cells. The subsequent appearance of many labeled cells in the pearls indicated that the development of pearls might be dependent upon the incorporation of labeled, undifferentiated cells in a field of differentiation evolving a pearl or that the further development of pearls might depend upon migration of stem cells or their progeny into the pearls, where they acquired the overt manifestations of differentiated squamous cells, as postulated by Frankfurt (6). Support for this idea was obtained from electron radioautographs in which the labeled cells from the 2-hr specimens were undifferentiated and those taken from the pearls at 96 hr were differentiated, with the cytoplasmic and membranous features typical of squamous cells. The end point of the differentiation of these squamous cells was nonviable.
keratin, as in the normal situation. It formed a central nidus in the pearl. We were not concerned with this nonviable material (except as it diluted the transplanted squamous cells); rather, focus was on the squamous cells, recognizable ultrastructurally as well differentiated, incapable of synthesizing DNA, and incapable of forming a tumor on transplantation. Thus it may be concluded that some of the progeny of malignant stem cells differentiated into postmitotic and benign squamous cells.

These experiments with different methodologies confirm the results obtained in studies of teratocarcinoma in which the stem cells proved to be multipotential, giving rise to many types of tissues (10), the majority of which were benign (20, 21), and our results are in accord with the experiments of Rabinowitiz and Sachs (23), who isolated epithelial variants from clones of cells transformed by polyoma virus. These variants had decreased cloning efficiency, loss of polyoma transplantation antigen, decreased tumorigenicity, and contact inhibition. These variant cells still contained polyoma genome, and the authors believe that they have reverted to benign cells. It is conceivable that these cells differentiated, as did those of the teratocarcinoma and squamous cell carcinoma.

In certain highly malignant monocellular tumors, the capacity for differentiation is much less, although ultrastructural evidence of differentiation resembling a caricature of spermatogenesis has been found in seminoma (18). In this situation, stem cells with a paucity of endoplasmic reticulum and Golgi profiles but with numerous polysomes and free ribosomes were present, as were cells with well-developed Golgi complexes, smooth endoplasmic reticulum, and proacrosomal granules representing differentiation toward spermatids. Many stages in development were observed between these extremes. In addition, spermatocytic seminomas have been described recently, which have even more complete differentiation, including the presence of intercellular bridges typical of spermatids (25). Using similar methodology, Friedmann and Berg (7) described stages in differentiation from embryonal myoblasts into long, striated muscle fibers in a rhabdomyosarcoma, and more recently Nameroff et al. (15) demonstrated in a skeletal muscle tumor that only the undifferentiated monocellular elements incorporated tritium-labeled thymidine and that the multinucleated myotubes developed from them. They concluded that muscle differentiation occurs by the same processes that operate in normal myogenesis. Thus data are slowly accumulating to support the notion that the variable histological appearance of a tumor is due to relative degrees of differentiation and not to relative degrees of dedifferentiation. Not all agree on this point. For example, Lin et al. (12) examined nasopharyngeal carcinomas with the electron microscope and identified 4 ultrastructural cellular types with numerous intermediate forms. The lack of differentiation in some of these cells was attributed to extreme dedifferentiation; the better differentiated ones were believed to have dedifferentiated less. These ideas are in accord with the notion of Oberling and Bernhard (16), who state "that the greater the degree of dedifferentiation of a cancer cell the greater the loss of membranous ergastoplasm." Although our data do not disprove the notion of dedifferentiation to account for the development of heterogeneity in tumors, they demonstrate an alternative mechanism, one that evolves benign cells.

There are a few other demonstrations of malignant cells becoming benign; Seilern-Aspang and Krotchwill (28) demonstrated that epidermal tumors in the newt have the capacity to undergo differentiation to normal epidermal cells, and Braun (1) demonstrated that shoots from teratomas of plants have the capacity to develop into normal fertile plants. Finally, a few cases considered medical curiosities because of their rarity are on record, in which neuroblastomas of man have apparently spontaneously differentiated into benign ganglioneuromas (2). Neuroblastomas of mice, which in vivo are poorly differentiated, develop neural processes and other features of ganglion cells when explanted in vitro (8, 27).

These data are all in accord with the idea that direction of differentiation of cancer cells may be possible clinically. Saffiotti et al. (26) have taken advantage of the modulating effect of vitamin A on squamous epithelium (4) to reduce the incidence of squamous metaplasia and squamous cell carcinoma of the lung in rodents given intratracheal carcinogen. It will be interesting to know if vitamin A can direct the differentiation of malignant squamous cells to benign glandular cells.

Silagi and Bruce (29) have shown that amounts of 5-bromo-2'-deoxyuridine too small to inhibit growth of melanoma cells in vitro cause an alteration of the morphology of the cells, which display contact inhibition and reduce tumorigenicity. Thus this agent modifies both differentiation and malignancy, leading the authors to the conclusion that these cellular functions are regulated by similar mechanisms.

The observations obtained in this study support those of Mendelsohn (14), who studied cell division and tumor growth using tritium-labeled thymidine and radioautography. He demonstrated that as many as 40% of the cells of certain tumors were nondividing or sterile and raised the question of whether or not the sterility of the cells was irreversible. On the basis of our studies, it may be concluded that the sterile cells are probably postmitotic differentiated cells. Since the differentiated state is notoriously stable, one is led to the conclusion that the loss of malignant attributes is irreversible.

A cancer is composed of a heterogeneous collection of cells. In addition to the various stem lines, as described by Makino (13), there are all of the fully and partially differentiated progeny of each stem line. Cloning of a tumor merely selects 1 of the clonal lines; it does not mean acquisition of a homogeneous population of cells, as demonstrated in the experiments with the teratocarcinoma. This is an important point for molecular biologists to appreciate in their attempts to obtain homogeneous species of cells when analyzing the molecular aspects of tumors. It is also important for oncologists to realize that the mechanism of progression (5) is probably a sorting out of these heterogeneous populations of tumor cells according to their ability to survive, proliferate, and differentiate under the conditions of their environment.

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Fig. 1. Transplantable squamous cell carcinoma from an Irish rat, illustrating undifferentiated cancer with mitoses (arrows) and 2 well-differentiated squamous pearls with keratotic centers. X 600.

Fig. 2. Radioautogram 2 hr after administration of thymidine-3H. The squamous pearl is not labeled; numerous undifferentiated cells are labeled. X 600.

Fig. 3. Radioautogram to illustrate a labeled nucleus (arrow) in a pearl 50 hr after the administration of a pulse of thymidine-3H. X 600.

Fig. 4. Radioautogram to illustrate labeled nuclei (arrows) in a pearl 50 hr after the administration of a pulse of thymidine-3H. X 600.

Fig. 5. Radioautogram with the electron microscope fixed 2 hr after a pulse dose of thymidine-3H. The labeled cells are undifferentiated stem cells. X 29,500.

Fig. 6. Radioautogram with the electron microscope fixed 96 hr after a pulse dose of thymidine-3H. The labeled cells are located in a pearl adjacent to the basal layer. The cell at the left is undifferentiated in comparison to the one at the right, which has tonofibrils, membrane-lining granules, and significant profiles of endoplasmic reticulum. X 16,000.

Fig. 7. Radioautogram with the electron microscope to illustrate a labeled cell in a pearl 56 hr after injection of a pulse of thymidine-3H. X 9,000.

Fig. 8. Higher magnification of outlined portion of Fig. 7 to illustrate the sophisticated differentiation of this cell. Note the desmosomes, microtubules, rough endoplasmic reticulum, and keratohyaline granules. X 40,000.

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