Perspectives in Cancer Research

Tumors as Caricatures of the Process of Tissue Renewal: Prospects for Therapy by Directing Differentiation

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Abstract

A concept of neoplasms, based upon developmental and oncological principles, states that carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells. The concept brings order to the facts about carcinoma, has predictive value for embryogenesis, and indicates possibilities for differentiation therapy. One such possibility assumes on the basis of experimentation in vitro that malignant stem cells can be induced to differentiate into postmitotic cells by application of chemicals. Another suggests study of naturally occurring substances which regulate cell proliferation and differentiation in adult tissues. The other possibility, based upon experiments in vivo and in vitro, indicates that embryonic fields are capable of converting their closely corresponding malignant lineages into apparently normal lineages responsive to homeostatic control. Induced differentiation of embryonal carcinoma has been achieved in vivo with improvement in longevity of the host and in some cases with apparent cure. However, ultimate success of treatment based upon turning malignant cells into benign cells will depend upon the nature of the benign cells. Will they remain benign?

Introduction

The idea of conversion of malignant cells to benign cells as a possible means of therapy for patients with metastatic carcinoma was proposed in 1961 (1). It was based on observations made upon teratocarcinomas of mice which demonstrated that some of the progeny of malignant cells spontaneously differentiated into benign if not normal cells (2, 3). These differentiations could be enhanced and apparently directed in tissue culture (4). Thus, contrary to dogma, cancer cells do not always beget cancer cells, and malignant cells may differentiate in response to environmental influences, although they are not responsive to normal homeostatic controls.

There are multiple reasons why the attractive idea of “differentiation therapy” did not “catch on” immediately. The mechanisms of differentiation and induction were and are poorly understood, and the critical observations were made upon teratocarcinomas, which are rare tumors erroneously viewed by many oncologists as not representative of tumors in general. Finally, clinical oncologists were beginning to have success with cytotoxic therapy.

Recently, interest in differentiation therapy was aroused in part by clinicians who noted that metastases in the lungs of patients with embryonal carcinoma sometimes failed to regress after cytotoxic chemotherapy, but neither did they grow and kill the host. Although these metastases slowly increased in size, they contained no cancer cells but only the mature adult tissues characteristic of teratoma (5). Apparently any cancer cells surviving chemotherapy had differentiated. Simultaneously, it was shown in vitro that embryonal carcinoma cells (6–9), erythroblastic leukemia cells (10–12), neuroblastoma cells (13), and leukemia cells (14–17) as well as other tumor cells (18–20) could be induced to differentiate by a variety of chemicals.

Is the further development of differentiation therapy, with its attendant effort and expense, warranted in view of the recent spectacular successes achieved with chemotherapy? The answer is yes, because there are two serious problems associated with cytotoxic chemotherapy. The first is that none of the cytotoxic agents developed thus far have specificity for malignant cells, and as a consequence normal cells are killed during treatment, causing morbidity and at times death of the host. The second problem is that most of the common cancers do not respond well to currently available chemotherapeutic agents.

The first international meeting on differentiation therapy was organized in 1986 by Dr. S. Waxman of New York, Dr. G. Rossi of Rome, and Dr. F. Takaku of Tokyo. Much of the discussion focused on the identification of chemicals that would direct malignant to terminally differentiated cells. Unfortunately, most of the effective agents under consideration were extremely toxic, the very attribute to be avoided. Naturally occurring growth factors, which may also modulate growth and differentiation of malignant cells, were also discussed. They are nontoxic and are in clinical trials. Because of the complexity of the problems associated with differentiation therapy, this appeared to be an opportune time to enlarge upon a concept of cancer that not only orders the facts of neoplasia (21, 22) but also clearly outlines avenues for differentiation therapy.

The Concept

The concept states that carcinoma is a caricature of the normal process of tissue renewal (21–23) as it might occur, for example, in skin, bronchus, intestine, testis, or bone marrow. The term caricature was chosen because its essential idea is a gross exaggeration of a normal characteristic. The overproduction of cancerous cells with their malignant attributes in relationship to the number that differentiates is the exaggerated characteristic that distinguishes cancer from normal tissue. A normal cell lineage is schematically represented in Fig. 1 (bottom). As a result of carcinogenesis, the normal stem cell produces an MS. Although some tumors have little or no capacity for differentiation, occasionally the offspring of others undergo terminal differentiation as illustrated in Fig. 1 (top). Unlike the normal lineage in which the stem cell divides to replace a senescent cell, in the cancer many more malignant stem cells are produced than differentiate. The cells of the mass do not cycle faster than normal cells, yet the mass grows rapidly because of the large number of cycling malignant cells.

Evidence supporting each aspect of the caricature in Fig. 1 will be considered now, and possible mechanisms for differentiation therapy will be presented.

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2 The abbreviations used are: MS, malignant stem cell(s); PC, precursor cells; CSF, colony-stimulating factor; G-CSF, colony-stimulating factor-granulocyte; M-CSF, colony-stimulating factor-macrophage; GM-CSF, colony-stimulating factor-granulocyte-macrophage; GMF, glia maturation factor; TGF, transforming growth factor; RA, retinoic acid.
The Cells of Origin of Carcinomas

The precise cell of origin of most tumors is unknown. Evidence for the idea that the stem cells of normal lineage are the target in carcinogenesis stems from studies of teratocarcinoma (33). Teratocarcinomas are composed of embryonal carcinoma cells and many mature tissues (skin, brain, muscle, cartilage, bone, glands, etc.), representing each of the embryonic germ layers (2, 3). The embryonal carcinoma cells, which have been shown to be the multipotential stem cells of these tumors, are extremely malignant (34). Moreover, the primordial germ cell, which can be conceptualized as the stem cell of the species, is the cell of origin of spontaneously occurring testicular teratocarcinomas (33). Thus, stem cells (S) in Fig. 1 can give rise to MS. Furthermore, the progenitor cell, PC, that in the colon gives rise to the several different types of colonic stem cells (S) (mucous, resorptive, and endocrine) also undergoes carcinogenesis, because colonic tumors exist that are composed of mucous, columnar, and endocrine cells (35). There are also colon carcinomas composed only of pure mucous, pure columnar, or pure endocrine cells (36). Therefore the normal stem cells of each of these lineages can undergo carcinogenesis independently of the PC. As mentioned previously, a similar situation exists in the hematopoietic system (30). It can be inferred from studies on leukemogenesis (37) that any cell capable of mitosis can undergo oncogenesis. Thus, for example, cell B of the normal lineage could become malignant and form a tumor the malignant stem cell of which would be B'.

Malignant tumors appear undifferentiated because many undifferentiated malignant stem cells are produced in relationship to the number that differentiate. It is unnecessary to evoke variable degrees of dedifferentiation as a mechanism to explain the varied appearance because the ultrastructure of primordial germ cells (the normal stem cell) and that of embryonal carcinoma cells (the corresponding malignant stem cell) are equivalent in terms of differentiation (38, 39). The same holds true for many systems.

The Caricature

Rather than representing a degree of dedifferentiation as was once believed, the well-differentiated cells of carcinomas have been proved to be derived by differentiation from the rapidly proliferating undifferentiated ones. For example, by administering tritiated thymidine to animals carrying a squamous cell carcinoma and following the label with time by autoradiography, it was evident that the keratinocytes of the squamous pearls of these tumors had differentiated from the undifferentiated malignant cells and were postmitotic and nontumorigenic (40). Thus, malignant cells even though aneuploid and with the other characteristics of anaplasia acquired during the process of progression (41) may arrive at the same end point in differentiation as the normal cell lineage.

The first demonstrations of differentiation of malignant cells were made on teratocarcinomas of mice (2), in which the embryonal carcinoma cells were separated from the mature tissues and were shown to differentiate into the various benign tissues of the teratocarcinoma (3). In these experiments, the embryonal carcinoma cells first proliferated and formed aggregates, which closely resembled early mouse embryos. They were named embryoid bodies (3, 4). However, with further proliferation of the cancer, the embryonic organization broke down and chaotic mixtures of tissue typical of teratocarcinoma resulted (3). This multipotency of embryonal carcinoma cells was confirmed by cloning the cells in vivo where they formed typical teratocarcinomas (34).
In these experiments the embryonic tissues derived from the cancer were benign and of no danger to the host (3). However, if the embryonal carcinoma differentiated into extraembryonic tissues, yolk sac for example, the clinical outcome was completely different. These extraembryonic tissues were always malignant (42). It is not known if the cancer-derived yolk sac was malignant from the outset or was benign but had a propensity for immediate transformation. A bone-forming cell line has been isolated from embryonal carcinoma, some cells of which form osteosarcomas on transplantation (43). If differentiation therapy is to be successful, it is essential that the tissues derived by differentiation of malignant cells be benign and stay benign.

Teratomas also occur in plants as redundant masses of rapidly proliferating disorganized cells with shoots, leaves, and rootlets. When the undifferentiated cells were cloned from these teratomas, typical teratomas were produced which contained an admixture of differentiated and undifferentiated tissues (44, 45). When the more normal looking tissues were cultured they developed into flowering plants that in some cases even set fertile seed. It was found that the plant teratomas synthesized auxin and kinetin, two essential growth factors, which are normally produced in embryonic plants but not in adult plants. Years later the production of growth factors by mammalian tumors was demonstrated (46, 47), but only recently have we realized the most important implications of Braun’s observation, “If a tumor makes a growth factor, that factor will be made by the cognate normal cell lineage during its development” (44). Study of these factors produced by tumors will give clues about embryonic regulation.

Studies of the leukemias also support the concept that tumors are caricatures of the process of tissue renewal. The first insights came from studies of Till and McCulloch (48) who developed the spleen colony assay in which the existence of a pluripotential hematopoietic stem cell (PC in Fig. 1) was eventually demonstrated. Bradley and Metcalf (49) and Pluznik and Sachs (50) independently developed methods for culture of leukopoietic tissues, and the interrelationship and differentiation of the various lineages was quickly established in vitro (30). The growth and regulation of these cells were dependent upon growth factors and factors for differentiation which will be discussed later.

An excellent review of differentiation-linked leukemogenesis (37) stresses that the phenotypic and enzymic markers of leukemia and lymphoma align with their normal counterparts in lymphopoiesis. In other words B’ has a normal counterpart in B in Fig. 1. Some of these phenotypes are rare and transitory and are present in higher frequency in regenerating marrow or fetal tissue (51). This makes good sense because of the rapidity of cell turnover under the circumstances of regeneration. Interestingly, no leukemia-specific antigens have been found. Some of the antigens used to identify subsets of leukemia cells turn out to be receptors for transferrin (52). Greaves (37) conceptualizes acute lymphocytic leukemia as frozen in the act of receptor gene assembly and expression.

Just as the squamous cell carcinoma is a caricature of the renewal of squamous epithelium (40), the leukemias caricature leukopoiesis and lymphopoiesis (51), and the embryonal carcinomas caricature the process of embryogenesis (42). The significant difference between these tumors lies in the potential of their respective determined stem cells. The stem cells of squamous cell carcinoma have had their potential reduced to one: the production of keratinocytes only. The multipotency of the embryonal carcinoma is equivalent to that of its normal counterpart, the inner cell mass of the early embryo (53, 54), which differentiates into the three germ layers. Cell lineages that develop later in embryogenesis can also be multipotent, as exemplified by hematopoietic tissues (51) and colonic mucosa (35).

From the examples discussed, it is clear that offspring of malignant cells have the capacity for partial if not complete expression of the differentiated phenotype according to the original determination of the cell lineage. Some tumors in the in vivo environment express none of the phenotypic markers of the tissue of origin, but like the imaginal discs, they still have the histiotypic determination of the tissue of origin, because when treated in vitro with retinoic acid or when placed in vitro and fed media containing retinoic acid, they express the potential for differentiation. Carcinogenesis does not alter the original histiotypic determination; rather, it superimposes the malignant phenotype on it. When malignant cells differentiate, they do so in accord with the histiotypic determination, and curiously the malignant phenotype is usually abrogated in the process. The mechanisms involved are not known.

The concept that tumors are caricatures of tissue renewal has predictive value. When a hitherto unidentified cell was observed electron microscopically in an adenocarcinoma of the colon of the rat, it was postulated that a similar cell should be present in the normal colon if tumors are truly caricatures of the process of tissue renewal (35, 55). Such a cell was found, and it was proved to be the agranular stem cell of an endodermally derived endocrine lineage of the colon (35). In addition, a line of embryonal carcinoma cells when injected into blastocysts differentiated into trophoderm (56). As mentioned, embryonal carcinoma cells are the neoplastic equivalent of inner cell mass cells. If the concept is correct, inner cell mass cells should have the potential to differentiate into trophoderm. The evidence is now overwhelming that in young blastocysts this is the case (57, 58). As mentioned above, monoclonal antibodies have been made to lines of leukemic cells as would be exemplified by cells A’, B’, etc. in the upper arm of Fig. 1. Leukemologists now take for granted that cells A, B, etc., in the normal lineage will have corresponding antigens identified by these monoclonal antibodies (51). Thus the concept allows identification of the place in the normal cell lineage where carcinogenesis occurred.

On the basis of Braun’s observation (44) that plant teratomas make growth factors that are active in the embryonic plant, it can be postulated that if a growth-regulating substance is made in a tumor, the same molecules will be made and serve a regulatory role in the development of the normal cell lineage. This may involve an autocrine mechanism and regulate the development of the lineage itself (59), or it may have a paracrine effect. The idea that regulatory molecules produced by tumors may give important clues to what goes on in the embryo has recently been supported by the observations of Rizzino and Bowen-Pope (60) and Rizzino (61). They not only have shown the production of growth factors in teratocarcinoma cells but have also demonstrated the same factors in the blastocyst.

In summary, it would appear that tumors are accurate caricatures of the process of tissue renewal and that observations made in tumors may have important implications for elucidating the development of cell lineages and their regulation in the embryo. With this understanding, it may be possible to develop new strategies for differentiation therapy.

Reversal of the Malignant Process

The classical example of clinical reversal of the malignant process was described by Cushing and Wolback in 1927 (62). A baby with advanced neuroblastoma was treated palliatively. Years later at appendectomy, the metastases of this malignant
tumor were found to have spontaneously differentiated into benign ganglioneuromas. This extremely rare phenomenon is known as spontaneous regression (63) and indicates that reversal of the malignant process can occur in vivo.

From a basic science standpoint, the observation that some of the offspring of malignant cells differentiate and become benign (Fig. 1) has also been construed by some as a reversal of the malignant process. This is not true. The malignant process is not reversed when at mitosis one of the offspring of a malignant stem cell remains as a malignant stem cell and the other differentiates.

Reversal of the malignant process would require the malignant stem cell (MS) illustrated in Fig. 1 to change into a normal stem cell (S) responsive to homeostatic controls. An experiment by Brinster (64) demonstrated just that.

Brinster injected embryonal carcinoma cells into blastocysts, and the injected blastocysts were placed in the uteri of pseudopregnant mice. One baby mouse was acquired that was chimeric in terms of coat color. Later, cancer-derived cells were demonstrated biochemically in almost all of the tissues of the offspring of blastocysts injected with embryonal carcinoma cells (65, 66). Apparently, the cells were induced by the blastocyst to behave like inner cell mass cells, and they and their offspring responded to the regulatory mechanisms of the embryo to populate the various tissues of these chimeric animals.

It should be noted that when embryonal carcinoma cells were first shown to differentiate into benign tissues, it was not known if the differentiated cells and tissues could respond normally to regulatory controls (2, 3). Cells of benign tumors do not. Thus, the significant contribution of the Brinster experiment (64) was to show that the offspring of embryonal carcinoma cells injected into the blastocyst were not simply benign tumor cells; they were normal cells in the sense that they responded appropriately to normal developmental and physiological stimuli. In other words, it showed that cell E' in Fig. 1, even if it has an abnormal karyotype, corresponds to cell E in its physiological responses.

From a standpoint of differentiation therapy, blastocyst regulation of embryonal carcinoma suggests than an embryonic field in which the normal lineage develops is capable of re-regulating the malignant stem cells derived from that lineage.

**Differentiation Therapy**

The concept that carcinoma is a caricature of tissue renewal indicates mechanistic points of attack for differentiation therapy. The first possibility, typified in the upper part of Fig. 1, is to direct the differentiation of all malignant stem cells of the caricature to terminally differentiated "normal" cells, a shift to the right. This was the most popular approach proposed at the First Conference on Differentiation Therapy. The second is to convert MS into apparently normal stem cells. Any of these objectives might be accomplished with chemicals, naturally occurring substances, or embryonic inducers.

**Embryonic Induction of Malignant to Benign Cell Lineages**

The working hypothesis is that inducers that allow a normal stem cell to be produced from its multipotent precursor in the embryo (PC to S in Fig. 1.) will also induce the histotypically related MS to reassume its role as a normal stem cell responsive to homeostatic control.

In support of the feasibility of this approach are the normal cell lineages induced in embryonal carcinoma by the blastocyst (64–66). Normal leukopoietic lineages have also been derived when mouse leukemia cells were injected into the placentas of 10-day mouse embryos (67, 68). Although most of the injected embryos died of leukemia, a not unexpected result since the tumor in question is extremely malignant, the astounding aspect of the experiment is that two surviving animals were chimeric in their leukopoietic tissues, as determined by glucose phosphate isoenzyme analyses. Apparently in these animals all of the injected leukemia cells differentiated and became innocuous (67, 68).

In other experiments, neuroblastoma cells (C1300) injected into the neural crest migratory route (69) and into the primitive of the adrenal gland (at the time normal neural crest cells migrate into it) do not form tumors or colonies in expected numbers (70). The fate of these injected cells is unknown. In contrast, melanoma cells (B16) also of neural crest origin were not regulated when injected via the neural crest migratory route. They formed tumors (71). However, melanomas did not grow in expected numbers when the melanoma cells were placed in embryonic skin on the day that normal pigment cell precursors arrive in the skin (71).

If four cancers of four tested can be regulated in terms of tumor formation by their appropriate embryonic fields, then it can be postulated that there is an embryonic field capable of regulating tumor formation of every carcinoma. Which embryonic field-neoplastic system will be most amenable for studying the mechanism of embryonic induction and thereby the mechanism of neoplastic regulation is unknown. The idea of using neoplastic cells to determine the mechanism of induction is a unique approach to understanding the mechanisms of early embryology, and thereby a basis for differentiation therapy.

In blastocyst regulation of embryonal carcinoma it has been shown that blastocoele fluid contains a soluble factor(s) which, in combination with contact of the embryonal carcinoma cell with trophoderm or inner cell mass, causes differentiation of the cancer cell (72). The effect is specific since melanoma cells, leukemia cells, and sarcoma cells are not affected by the blastocyst (73). There is evidence that the effect of embryonic skin on melanoma cells is mediated by a small molecular weight factor synthesized in a narrow window of time (71). This factor causes retardation of growth of melanoma cells in vitro. Large doses kill melanoma cells.

Nothing is known regarding the mechanism of regulation of leukemia cells in the placenta (67, 68) or of neuroblastoma in the neural crest migratory route (69).

Whether induction in normal systems is mediated by cell contact, by a diffusible factor, or by a combination of both is not known. In the prototype experiment, presumptive mesenchyme (dorsal lip of blastopore) when placed beneath ectoderm of amphibian embryos evoked a new embryonic axis (74). Grobstein (75) demonstrated induction of tubules in various epithelia cultured on opposite sides of a trans-Millipore filter from mesenchyme and believes that a diffusible molecule, possibly a mucopolysaccharide, mediates this differentiation. Saxen et al. (76), however, believe that transfilter differentiations are mediated in part by cell processes which penetrate the filter membranes, while Levine et al. (77) have isolated a soluble factor from inducing mesenchyme which alone causes differentiation in the epithelium. Cunha and Lung (78) showed that adult bladder epithelium of rodents when cultured with urogenital sinus mesenchyme (presumptive prostatic connective tissue) differentiated into glandular epithelium and synthesized prostatic markers. When human bladder cancer was mixed with rodent urogenital sinus mesenchyme, it also assumed a prostate-

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1. G. B. Pierce, A. Lewellyn, and R. E. Parchment. Inner cell mass in the presence of blastocoele fluid regulation colony formation by the blastocyte, manuscript in preparation.
like morphology (79). The biochemistry and molecular biology of these reactions remain to be elucidated. The problem is clearly outlined in a recent review (80).

Studies of differentiation at phenomenological levels indicate that the process requires specific tissues, cells competent to react, and finally the presence of inductive signals (81). For example, guinea pig bone marrow is a potent inducer of mesodermal structures in *Triturus* (82). Furthermore, factor isolated from 7-day-old chick brain is a potent inducer of neural tissue (83), and extracts of liver induce archencephalic structures in amphibian embryos (84). Thus it may be concluded that inducers produce their effects across species boundaries and embryonic tissue may not be their richest sources. Finally, it appears that regulation of epithelia may be mediated by mesenchymal tissues.

**Induction of Differentiation with Naturally Occurring Substances**

Of the naturally occurring substances that influence growth and differentiation, those of the lymphoid (37) and leukopoietic tissues (85, 86) have been most widely studied. Attention will be paid only to colony-stimulating factors, glia maturation factor, and tumor growth factors. Excellent reviews are available on most of these factors (85–87) and only points germane to this “Perspective” will be considered.

**Colony-stimulating Factors.** The CSFs are a series of at least four glycoproteins, with a half-life of less than 15 min, synthesized in minute amounts in most if not all tissues (85). They are essential for the survival and growth of hematopoietic cells in vitro (49, 50) and presumably regulate hematopoiesis in vivo. They act in a dose-responsive manner via receptors on cells at various points in the scheme of hematopoiesis (86).

Multi-CSF is the only known stimulator of proliferation for the progenitor cell of the hematopoietic system (colony-forming unit-spleen) which differentiates into the stem cells for each of the megakaryocyte, erythrocyte, granulocyte-macrophage, and lymphocyte lineages (88). Each of the latter stem cells is determined for its particular lineage, and each is stimulated to divide by multi-CSF. This factor acts locally, is not present in the circulation, and appears to be identical to interleukin 3 (89). Two other CSFs, G-CSF and M-CSF, regulate growth of granulocyte and macrophage lineages, respectively (88, 90), and the final one, GM-CSF, is responsible for regulation of granulocytes and macrophages (85, 91).

Differentiation of granulocytes and macrophages is also induced when GM-CSF is added to media of acute leukemias in vitro (85, 91). G-CSF also suppresses leukemia stem cells and induces terminal differentiation (88). G-CSF, GM-CSF, and multi-CSF have now been cloned.

Like normal cells most strains of leukemia cells require colony-stimulating factors for survival and proliferation in concentrations comparable to those for normal cells. Some leukemic cells synthesize colony-stimulating factors but the amounts are small in comparison to those produced by normal cells. GM-CSF and G-CSF in vitro can induce cells in a culture of myeloid leukemia to differentiate into granulocytes and macrophages (a shift to the right), but multi- and M-CSF appear to stimulate growth only (85, 91).

As with the teratocarcinomas, interesting possibilities for developmental biology and oncology may be derived from these bits of information. Sachs et al. (67, 68) demonstrated regulation of leukemic stem cells by the embryo with the production of normal leukopoietic lineages. In this situation MS of Fig. 1 became S as a result of embryonic induction and it and its progeny were regulated by CSFs to produce an animal chimeric in leukopoietic tissues. It is not known if CSFs might also act as embryonic inducers and convert malignant stem cells into benign stem cells responsive to homeostatic control. Whether or not these agents prove useful in differentiation therapy remains to be seen. They are useful clinically in stimulating leukopoiesis.

From a therapeutic standpoint, it does not matter if differentiation therapy shifts cells to the right or induces them to become normal stem cells, unless the “normal” stem cells easily revert to the malignant phenotype.

An interesting analogy is to be had in pernicious anemia. Patients with pernicious anemia have an overgrowth of abnormal erythrocyte precursors, with colonies of these cells in the liver and spleen. If untreated the patients invariably die, but if they are treated with vitamin B12 the morphology of the cells returns to normal and the patients survive as long as the treatment is maintained. The importance of the analogy is that some therapeutic agents may affect differentiation of MS in Fig. 1 only as long as they are present, whereas other agents will cause a shift to the right causing terminal differentiation, and yet others may cause MS in Fig. 1 to become S, a normally responding cell lineage.

**Glia Maturation Factor.** GMF, an acidic protein isolated from mature brain tissue, is capable of promoting growth and differentiation of fetal astroblasts in culture. Both morphological and biochemical markers of differentiation have been shown to be induced by GMF including formation of glial filaments and increased production of S-100 protein, glial fibrillary acidic protein, and several enzymes (92).

There is a dual effect of GMF on glial tumor cells in vitro. Subconfluent cultures are growth stimulated whereas confluent cultures become arrested and contact inhibited (93). GMF inhibits the growth of rat C6 glioma cells grown as s.c. tumors in nude mice (93). This is associated with morphological changes and increased production of glial fibrillary protein indicating the induction of differentiation. A human glioma cell line (HG-1) was also shown to differentiate in vitro when GMF was incorporated in the media (93). It is important to note that the developing system may not be the richest source of regulatory molecules as exemplified by adult brain as the source of GMF. In addition, guinea pig liver and spleen are good sources of neural and mesenchymal inducers in amphibians.

**Transforming Growth Factors.** TGFs are found in media conditioned by tumors. They stimulate fibroblasts to proliferate and assume a transformed phenotype in soft agar (94–96). They are considered to be an essential part of the neoplastic state because they are synthesized by cells transformed by temperature-sensitive mutants of the Rous sarcoma virus only during the permissive temperature (97). They function in autocrine and paracrine modes and are potent stimulators of angiogenesis. They have not been described in hematological malignancies but are produced by normal embryonic cells (61).

TGFα is present in minute amounts and stimulates growth by binding to epidermal growth factor receptors of cells (98). It has about a 35% homology with epidermal growth factor. TGFβ and platelet-derived growth factor cause transformation of rat fibroblasts in vitro (99).

TGFβ is not related to TGFα (87, 100–101). It is synthesized by normal embryonic and adult cells as well as by neoplasms. Under some conditions it stimulates growth of cells, and under others it inhibits growth and causes differentiation. Serum has been reported to have differentiating promoting effects on some cells, an activity attributable to TGFβ released by platelets in the coagulation process which produces the serum. In particular
TGFβ induces squamous differentiation and seems to stabilize that differentiation. On the other hand it blocks the differentiation of T-lymphocytes.

Nothing is known about a possible role for tumor growth factors as possible regulators of malignant growth and differentiation at the clinical level. What is important is that these agents are made in the embryo at the time the normal lineage is developed. In the embryo they must play autocrine or paracrine roles in regulation, whether alone or in conjunction with other molecules.

**Induction of Differentiation with Chemical Agents**

The objective of chemical therapy is to direct all malignant stem cells and their malignant descendants to postmitotic cell types in a manner nontoxic to the host. It is also conceivable that MS could be converted to normal stem cells by chemical treatment, but that has not been demonstrated to date.

There are two excellent reviews of clinical and basic research in chemically induced differentiation of neoplastic cells (103, 104). These reviews have comprehensive accounts of the agents tested to date. Of these only hexamethylene bisacetamide, retinoic acid, 5-azacytidine, and 1-β-d-arabinofuranosylcytosine will be briefly considered here because they illustrate specific points in differentiation therapy.

**Hexamethylene Bisacetamide.** Hexamethylene bisacetamide enhances differentiation in a variety of tumors (106–108); its effects on the differentiation of the erythroleukemia of mice induced by the Friend virus are most illustrative of its action. Erythroleukemia cells grow well as established lines in tissue culture, and some produce hemoglobin (12), yet even after prolonged culture they produce tumors when injected into appropriate hosts (108). The cells are considered to be blocked in the process of maturation.

Hexamethylene bisacetamide added to tissue cultures of erythroleukemia cells converts most of the cells to hemoglobin production and a terminally differentiated state within 48–60 h of treatment. There are changes in cell volume (109) and membrane fluidity (110) and in the expression of protooncogenes (111–113). The data suggest that malignant stem cells in Fig. 1 were shifted to the right in their differentiation. There is no evidence that MS were converted to normal stem cells. The latter is not ruled out, however, because the tissue culture system may not have been appropriate to demonstrate such a shift.

Much needs to be done in testing this agent. Is it effective in low doses that will reduce toxicity? Can clues be obtained about its mechanism of action which might allow search for similar but more effective agents?

**RA.** RA enhances differentiation of a variety of tumors in vitro (104). The effects on embryonal carcinoma are illustrative (114). When grown on plastic, embryonal carcinoma cells reproduce only themselves, but in the presence of RA embryonal carcinoma cells differentiate into endodermal cells (114). These do not revert to embryonal carcinoma. By changing regimens of RA, many tissues differentiate from the embryonal carcinoma (7), as measured morphologically (7, 115), biochemically (116), and immunohistochemically (114–116). In some instances “incomplete” differentiation has been observed, but whether this represents the effects of mutation, modulation of response, or incomplete treatment is not always known (116–119). Receptors for RA have been described on embryonal carcinoma cells (120). It is conceivable that resistance to therapy in some situations may reflect a lack of receptors.

An embryonal carcinoma was also used in the first successful experimental attempts at differentiation therapy of a solid neoplasm (9). RA in pharmacological amounts was administered to animals bearing transplants of embryonal carcinoma. The experiments were started when the tumors reached a diameter of 5–10 mm. The control groups of animals were treated with the vehicle in which the RA was administered. Control animals all died from the growth of the tumors in a short period of time, and when examined at autopsy the tumors proved to be undifferentiated embryonal carcinomas. The tumors of all animals treated with RA had differentiated to a degree although most animals died as a result of growth of their tumors and treatment with RA. In the RA-treated animals that survived the tumors grew slowly. After several months, they contained no embryonal carcinoma cells, only the differentiated tissues characteristic of benign teratoma. When these tumors were transplanted, the vast majority failed to grow. However, 2 of 11 grew as malignant tumors although neither was an embryonal carcinoma: one was a glial tumor; the other contained a mixture of glial and cartilagenous cells (9).

Important observations result from this study: (a) in the mice that survived RA toxicity, differentiation therapy was successful; (b) not all phenotypically differentiated cells derived from the cancers were benign. Little is known about the nature of cancer-derived “normal” cells. Are they apparently normal but initiated cells that might respond to tumor promoters? Are they noninitiated cells but more sensitive to carcinogenesis? Are they the equivalent of transgenic cells carrying an SV40 viral transcript (121) in that they are usually normal while integrated in the host but when placed in vitro behave as transformed cells? These questions are currently being studied, and the answers will shed light on the ultimate success of differentiation therapy. Finally, literally nothing is known of the cellular and biochemical mechanisms of RA therapy. Is there primarily a shift to the right (Fig. 1), or have MS been converted to normal stem cells, as the data from these experiments might indicate (9)? It is conceivable that the biochemical pathway for differentiation can be modified by a variety of chemicals at various points in the pathway thereby enhancing or inhibiting the process. The stimulation of the hypomethylation of DNA by 5-azacytidine discussed below is a case in point.

**5-Azacytidine.** 5-Azacytidine has a dramatic effect on the differentiation of mesenchymal cells and it is mentioned briefly to illustrate the intriguing insights that have been made in understanding its mechanism (122). The DNA of treated cells becomes hypomethylated and the cells differentiate into fat, muscle, and cartilage, in fact, into all of the differentiated derivatives of mesenchyme (123). Since no tissues representing the other two germ layers are produced, treatment does not appear to affect the mechanisms that determine the heritability and stability of the individual germ layers.

5-Azacytidine has been used to treat a patient suffering from β-thalassemia (124). γ-Globulin production increased 7-fold, and erythropoiesis became more effective as evidenced by an increase in hemoglobin and a reticulocytosis. Hypomethylation of marrow cells was also demonstrated. The risks of long term treatment are not known, which is extremely important because the effect of treatment was short lived (124). In addition, 5-azacytidine induces differentiation in Friend erythroleukemia cells by inhibition of DNA methyltransferase (125).

In summary, the studies of the action of 5-azacytidine have provided interesting information on the differentiation of mesenchyme-derived cells. A correlation has been shown between differentiation and hypomethylation of mesenchymal cells. This information will be important in understanding the mechanism of differentiation.
1-β-D-Arabinofuranosylcytosine. 1-β-D-arabinofuranosylcytosine is an agent used in the treatment of nonlymphocytic leukemia. In addition to its cytotoxic action it has been shown to induce differentiation of myeloid leukemia cells in vitro (126, 127). The mechanism of action is not clear (85). Baccaroni and Tura (128) treated eight leukemic patients with low dose 1-β-D-arabinofuranosylcytosine. Five of these patients had a significant increase in the proportion of blast cells with cytoplasmic granules, a morphological marker of myeloid differentiation. In another, dysmyelopoiesis was corrected in part because blast cells were reduced in number and the severely neutropenic state returned to normal. Subsequently conflicting reports have appeared either supporting (129–133) or refuting (134, 135) the possibility that 1-β-D-arabinofuranosylcytosine acts by induction of differentiation of leukemic cells.

The clinical response to low dose 1-β-D-arabinofuranosylcytosine is different from that of high dose cytotoxic therapy. There is a slowly evolving remission without an aplastic phase (129). Occasionally patients refractory to high dose 1-β-D-arabinofuranosylcytosine will respond to low doses, which suggests a noncytotoxic mechanism of action (130). Low dose 1-β-D-arabinofuranosylcytosine is also cytotoxic to a degree (131), but the evidence suggests that in addition there is a differentiating effect on the leukemic cells.

Concluding Remarks

The concept that tumors are caricatures of the process of tissue renewal brings order to the cellular facts of neoplasia and thereby lays the basis for extrapolating data from molecular studies of neoplasia to the development of normal cell lineages and vice versa, extrapolating data from molecular studies of embryology to the development of neoplasms. The reason for the overgrowth of malignant cells in relationship to the number that differentiate is not known, and this lack of knowledge highlights the essential problem in neoplasia which is to explain the stability and heritability of the neoplastic state.

The caricature points to two avenues for differentiation therapy. The first proposes that inductive environments responsible for the generation of determined stem cells in the normal lineages will be able to regulate the malignant stem cells of those lineages to the point that they and their offspring will respond to normal homeostatic mechanisms. It is not known why histiotypic differentiation of a malignant cell usually results in abrogation of the malignant phenotype. Although none of the molecular mechanisms are known, control of embryonal carcinoma cells by blastocysts requires both a soluble factor in blastocoele fluid and contact of the cancer cells with blastocyst cells. In the case of regulation of melanoma, a soluble factor produced by embryonic skin has been identified. This factor in high dose kills melanoma cells.

It makes sense to examine induction and differentiation in the embryo as a means of reregulating malignant cells. Whether or not any of the information will be useful in differentiation therapy remains to be seen. If malignant cells can be induced to become "normal" stem cells, much will need to be learned about the biological nature of cancer-derived cells before this form of differentiation therapy could be used safely. Naturally occurring substances that probably work by shifting differentiation to the right could be as effective therapeutically as inducers. It is not known if they might be embryonic inducers and make MS into normal stem cells.

The second avenue of approach proposes to direct differentiation of malignant to terminally differentiated cells using chemicals. Extensive in vitro studies support the feasibility and the mode of attack. The ones described in this paper are state of the art and not only show promise for differentiation therapy as a practical possibility but also provide intriguing possibilities for the understanding of differentiation itself. More work must be done in vivo, with focus on identification of agents less toxic than those currently used. In addition, chemicals for differentiation therapy may carry a peculiar toxicity of their own. If they are able to cause the differentiation of malignant stem cells, they may also cause normal stem cells to differentiate and thereby interfere with tissue renewal. This would be as devastating to the host as would be the outright destruction of these essential cells.

Finally, the nature of the cancer-derived "normal" cells derived by differentiation therapy must be determined. Not all of the tissues derived from embryonal carcinoma are benign. It will be interesting to see which of the embryonic inducers, naturally occurring substances, chemical mediators, or combinations of these approaches will be successful. The future looks bright for differentiation therapy.

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References


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