Biology of Cancer and the Cancer Cell: Normal and Abnormal Regulation of Cell Reproduction

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The distinguishing property of the cancer cell is its capacity to multiply and invade in situations where normal cells are restricted. The extraordinary research effort of the last decades to translate this primary difference between cancer and normal cells into unique and specific biochemical differences is based on the very reasonable premise that the discovery of a unique biochemical property for all cancer cells is virtually an essential step in the eventual design of completely and absolutely specific anticancer chemotherapeutic agents. Unfortunately, the efforts to achieve such translation have so far largely failed, but in the course of this "failure," an enormous amount has been learned about cell chemistry, physiology and reproduction.

We have learned to make a sharp distinction between abnormal cell reproduction and abnormal regulation of cell reproduction. The failure to recognize this difference has sometimes been a source of confusion, has inspired some fruitless research and has led to pointless controversy. Neoplasia is defined as the formation of any new and abnormal growth, but there is no evidence that in a neoplasm the cells grow and divide by any other than the same rules and mechanisms by which cells grow and divide in any normal tissue or in any other situation (e.g., in culture).

Thus, the abnormality in neoplasia is present not in the processes that con-
stitute growth and division, but in some degree of loss of regulation of cell reproduction. The degree of loss of such regulation is, from one type of neoplasm to another, highly variable but apparently rarely complete. This is so because neoplastic cells, with few possible exceptions, do not reach the high rates of proliferation that are quite usual for certain normal cell types (e.g., stem line cells in blood-forming tissue, or progenitor cells in certain epithelia).

Various hypotheses have been evolved based on the presumption that growth and division in a neoplastic cell are in themselves intrinsically abnormal, or that neoplasia results from altered patterns of such housekeeping activities as energy metabolism or deoxynucleoside triphosphate synthesis, but such hypotheses are clearly not in keeping with the specific experimental findings. In addition, they are not consonant with the present large body of knowledge about the biochemistry, genetics, physiology and cytology of cell reproduction. Neoplasia is the result of a heritable lesion or defect in the still unidentified mechanism that regulates the initiation of chromosome replication (i.e., the entry of a cell into the synthesis of deoxyribonucleic acid—DNA).

In cancers, the variable degree of loss of control over cell reproduction, observed as different growth rates, is accompanied by two additional, primary changes: (1) a failure to a greater or lesser degree of the cells involved to develop or maintain a fully differentiated, mature state; and (2) a loss of repression of cell migration that is expressed to a greater or lesser degree as an ability to invade normal tissues. A variety of observations point up the specific inter-relatedness of these three phenomena (i.e., loss of regulation of cell proliferation, a deficiency in differentiation, and a loss of repression of cell migration), but the elucidation of the molecular mechanisms that underlie the causality of this inter-relatedness is still to be reached. All three of these basic changes in cell behavior may be initiated by a single common causative event. It is well known that the more highly differentiated the cancer cell, the more slowly it grows, and vice versa. This is one of the observations that supports the thesis that an integral part of differentiation is the establishment of a rigid control system over cell reproduction. Incomplete differentiation of a cell—for example, of an hepatocyte—means not only absence of some normal hepatocyte properties, but includes an incomplete differentiation of regulation of cell reproduction. The greater the deficiency in differentiation, the greater the deficiency in the regulation of cell reproduction.

In basic research in cell biology, the study of the loss of regulation of cell reproduction logically proceeds from a prior understanding of the molecular basis for the intact regulatory mechanism in normal tissues. This latter understanding depends, in turn, upon a firm and detailed knowledge of the processes that constitute cell growth and cell division. The discussions in this paper deal, therefore, with the components of cell growth and reproduction that make up the life cycle of every proliferative cell, normal or abnormal. On this basis we proceed to the normal interruption of the cell life cycle by which the regulation of cell reproduction is accomplished. And finally, we consider briefly the possible basis for the loss of such regulation.

The Cell Life Cycle

All of the events of cell reproduction are encompassed within the period called the cell life cycle. The life cycle extends from the completion of one cell division to the completion of the next, and it contains all of the events necessary to produce a doubling in the structural elements and functional capacities of the cell, and—what is espe-
cially important in this discussion—it includes those events that are particularly and directly preparations for cell division. For present purposes, relatively little attention need be given to growth or development of the cytoplasm, because the pace of cell progress through the life cycle is governed principally by the nuclear events of chromosome replication and segregation (mitosis). All other growth activities are ultimately geared directly or remotely to the accomplishment of these chromosomal processes, and the regulation of cell reproduction is precisely a question of the regulation of chromosome replication.

The cell life cycle, which is divided into four subsections on the basis of chromosome replication and segregation, is summarized in the diagram. (Fig. 1.) The first subsection, which occupies the first part of interphase, is called the G1 phase, and it extends from the completion of the previous cell division to the beginning of chromosome replication. The essence of chromosome replication is deoxyribonucleic acid synthesis, and the beginning of DNA synthesis defines the end of the G1 phase and the initiation of the S (synthesis) phase. The S phase is usually six to eight hours in mammalian cells, and is followed by an interval of usually two to three hours, called the G2 phase, which separates the end of chromosome replication (DNA synthesis) from the detectable beginning of prophase in mitosis and cell division. The period of mitosis or division is called the D (division) phase and commonly lasts less than an hour. The terminology of G1, S, G2 and D was originated by Howard and Pelc¹, who first clearly defined the existence of the four subsections of the cycle from studies on plant root cells, although it had been shown previously by others that DNA synthesis occurs during interphase.² Although much has been learned about the properties and contents of all four of the cycle subsections, the definitions of the G1 and G2 phases continue to be essentially negative (i.e., no specific molecular events have yet been discovered which define or explain the existence of these two phases). They stand as time gaps between the clearly defined D and S phases.

The transition of a cell from G1 to S is a critical point in the cycle. Once DNA synthesis begins, a cell ordinarily proceeds quickly to the completion of division and the two resultant daughter cells enter the next G1 phase. Cells do not normally cease reproductive activity by becoming arrested in either S, G2 or D, although, in certain circumstances, both S or G2, and even D, can be very long. Because control of cell reproduction is essentially always achieved by action in the G1 phase, and never in either the S or G2 phases, little more will be said about these latter two. There are many important unanswered questions about events within S and G2, but these are not immediately germane to the problem of regulation of cell reproduction. In mammalian cells, G2 is probably concerned primarily with synthesis of the macromolecules needed for assembly of the mitotic apparatus and with the beginning of condensation of the chromosomes into their mitotic configuration.

The individual cell cycle is made up of a succession of many steps. The problem is to determine the nature of these steps and the means by which these steps are held together by cause and effect relationships in a way to produce the ordered continuity known as the cell cycle. The identification of the four major subsections of the cycle (i.e., G1, S, G2, and D) represents the beginning of the decipherment of this cause and effect sequence. It is likely that only a small fraction of the total genetic apparatus of a cell is concerned with these matters of continuity of the cell life cycle.
The preceding section presents a general view of the composition of the cell life cycle. The sections to follow deal with certain aspects of the cell cycle with particular attention to possible mechanisms for regulation of cell reproduction.

**The G1 Phase**

As already pointed out, the presence of the G1 phase is not defined by any known events but is the time gap between the end of cell division and the initiation of DNA synthesis. Considerable information has, nevertheless, been obtained about its properties.

The existence of G1 has sometimes been ascribed to preparations for DNA synthesis; in particular, the synthesis of enzymes needed to produce pools of the immediate precursors of DNA—i.e., the four deoxynucleoside triphosphates, and the synthesis of DNA polymerases and possibly other proteins concerned with configurational modifications of DNA in connection with replication. Some of these enzymes are, in certain cells, synthesized just before the initiation of DNA synthesis, an observation that supports the hypothesis already stated that a sequential cycle of gene activations underlies the cycle. But, at the very most, only the terminal part of G1 can be explained by these enzyme syntheses, and the explanation for the existence of G1 needs to be sought in other events.
Experiments with inhibitors have shown that both RNA and protein syntheses are necessary for a cell to progress through the G1 phase, but this type of information is too nonspecific to afford insight into the reasons for G1. Measurements of cell mass in mouse cells in culture have shown that the larger the cell at the beginning of G1, the shorter the subsequent G1 period. The measurements demonstrate that the mass of the cell, or some component of cell mass, has an important bearing on the rate at which the G1 events are accomplished, but unfortunately it does not contain any specific clue about the nature of these events. This relationship between cell mass and the length of G1 may be interpreted to mean that G1 is principally a period of cell growth, with the corollary that the mechanism that activates DNA synthesis is itself sensitive to total cytoplasmic mass or, more likely, to some specific part of cytoplasmic mass; for example, the amount of a particular protein. This important corollary is borne out by the observation in these experiments that in a population of cells there is less variation of mass among those cells initiating DNA synthesis than among those cells beginning the G1 phase. This suggests that initiation of DNA synthesis is related to the attainment of a given cell mass or a given amount of some particular part of cell mass.

Any explanation of the G1 phase must take into account the fact that a measurable G1 phase can be absent in the life cycle of some cells. For example, ascites tumor cells in mice have no G1 period under certain conditions permitting rapid growth, and rapidly proliferating stem line cells of erythropoieses may have little or no G1 phase. Cells in early cleavage stages in at least some animals lack G1, although G1 becomes a typical part of the life cycle for almost all cells of the embryo at later stages of development. Finally, a line of hamster cells has been described that grows without a G1 period. This is a particularly interesting example because this hamster cell line was almost certainly derived by a basic (genetic?) change from a hamster cell in which G1 was a regular feature. Perhaps it might be tentatively concluded that under conditions that favor very rapid cell proliferation, the cell cycle is shortened by reduction of the G1 phase to an immeasurably brief interval, or eliminated altogether. Unfortunately, we have no solid clues as to the underlying cause of G1 elimination.

Whatever the reason that G1 is present under some conditions and not under others, the observations reveal the important fact that G1 is expendable as a time period with respect to maintenance of continuity of the cell life cycle. In general, the facts are consistent with the proposition (developed below) that the G1 period is brought about by the controlled interruption of the cell life cycle—an interruption by which regulation of cell reproduction is achieved.

The most striking property yet discovered for the G1 phase is its variability in length within a population of cells of any given type. This was first clearly noted for clonal populations of cells proliferating in culture with a constant average cell cycle time. All cells show cell cycle times that are normally quite variable even though the average generation time for the population remains constant. The variability is due primarily to variation in the length of the G1 phase with relatively little or no fluctuation ordinarily present in the lengths of the S, G2 or D phases. The observation has been extended to situations in which the average generation time for cells of a uniform type can be lengthened by a shift to slightly less favorable culture conditions. In such cases the increased generation time is accounted for largely, if not entirely,
by an expansion of the $G_1$ phase with little or no increase in the durations of $S$, $G_2$ or $D$. This is not to say that increases in $S$, $G_2$ or $D$ do not occur under some conditions, but the major change is always in the length of $G_1$. An extreme extension of $G_1$ occurs in cells in culture that cease proliferation during stationary phase of culture growth; all the cells under stationary phase conditions are arrested in the $G_1$ state.

Especially pertinent is the arrest in $G_1$ of mammalian cells in culture as a consequence of contact inhibition. It is a well-established observation that normal (diploid) mammalian cells in culture cease migration and cell division when the cells become so crowded that large areas of contact between cells are established.

**Regulation of Cell Reproduction**

During cleavage stages of development, cell reproduction is extremely rapid. During this period of minimum restraint on cell reproduction, the cell life cycles are foreshortened by the elimination of any measurable $G_1$ period. At a point in the development of the embryo not yet determined, the rate of cell reproduction begins to decline in various groups of cells that have entered very early differentiation; there is some evidence that the reduction in reproductive rate is achieved by the introduction of a $G_1$ phase into the cells cycles. Finally, in the adult organism the degree of restraint operating on cell proliferation becomes stabilized, although it differs enormously from one tissue to another in a way that conforms precisely to the need for new cells to replace those lost by death. The degree of restraint in each tissue is exactly reflected in the time that the average cell requires to complete one life cycle. Average life cycle times, therefore, vary from many thousands of hours—in a very slowly renewing tissue such as liver—to 12 or less hours in stem line cells serving the very rapid turnover of erythrocytes.

The normal demand for new erythrocytes in man is roughly 2.5 million per second, which means the completion and initiation of 2.5 million cell life cycles every second.

The dynamic state of the regulatory mechanism that maintains the different and appropriate rates of cell reproduction in each adult tissue is dramatically apparent in the changes in the rates of cell reproduction brought about by wounding of epithelia, partial hepatectomy, acute loss of blood cells, or similar disturbances to renewable tissues. The rate of cell proliferation is greatly accelerated in such cases of acute cell loss until the cell population is restored to the normal level.

The analysis of the life cycles for cells in a variety of renewing tissues has produced an important insight into the normal regulatory mechanism. For example, in the alimentary tract of the mouse, the average cell cycle time for the epithelial stem cells varies from 17 hours in the ileum to 36 hours in the colon, to 85 hours for buccal mucosa, to 181 hours in the esophagus. It is a striking fact that these differences in cell cycle times are due to a change in the duration of only the $G_1$ period of the cycle. The combined duration of the $S$, $G_2$, and $D$ phases is approximately 10 hours in all cases, while the duration of $G_1$ varies from approximately seven hours in the ileum, 25 hours in the colon, 75 hours for buccal mucosa, to 171 hours in the esophagus. Under some circumstances, such as suboptimal nutrient conditions (for example, because of a poor blood supply), the lengths of $S$, $G_2$ and $D$ may be increased. But it is clear that the regulation of the rate at which tissue cells are allowed to reproduce is exercised by the retention of cells in the $G_1$ state for a shorter or longer period. Cell-cycle analyses are now available for the cells in a number of normal renewing tissues as well as in neoplasms, and they
bear out the generalization. Finally, in nonrenewing tissues of the adult (e.g., skeletal muscle cells and neurons) the differentiated cells are permanently arrested in the G₁ state.

This role of G₁ in the regulation of cell reproduction in renewing tissues and neoplasms is in striking accord with the established fact that in free-living cells of all types and mammalian cells grown in culture the natural arrest point for cell reproduction is in the G₁ phase. On this basis, it is reasonable to postulate that the regulation of cell reproduction in multicellular organisms has evolved out of the mechanisms by which cell reproduction is curtailed in free-living cells under natural (but unfavorable) conditions.

The discovery of this property of the G₁ phase represents a major and most significant advance in our understanding of cell reproduction and has brought into much sharper focus the whole problem of the regulation of cell reproduction and, most important, the loss of such regulation in neoplasia.

In this regard, it is pertinent to bring up once more the phenomenon of contact inhibition. We have very little idea of why or how contact between cells prevents their entrance into chromosome replication, but it is a most impressive fact that these cells can be released from inhibition by transformation with certain RNA or DNA viruses (e.g., polyoma virus, simian virus 40, Rous sarcoma virus). Viral transformation of diploid, normal cells is measured as a release from contact inhibition of both migration and reproduction. One of the first signs that transformation has occurred is the exodus of cells from G₁ with the initiation of DNA synthesis. Shortly following the initial events of viral transformation, the cells undergo a pronounced karyotype change from diploid to aneuploid.

The changes that occur in transformation (i.e., release from inhibition of migration and reproduction and development of aneuploidy) parallel strikingly the changes that convert a normal cell into a neoplastic cell. Because transformation can be induced not only by viruses but by radiation or carcinogenic chemicals, and because at least some cells transformed in culture are capable of producing tumors when injected into animals, it can hardly be doubted that cell transformation in culture is essentially the same process by which cancer develops in vivo.

The breakdown in the control of cell proliferation must, as a corollary to the discovery of the G₁ arrest, or G₁ extension of the cell cycle, stem from a loss of sensitivity in one or more G₁ events to a tissue-specific regulator substance (co-repressor). (Fig. 2.) Such regulator substances are still undefined in spite of a hard search, but a variety of observations on normal tissue development, experiments on partial hepatectomy, unilateral nephrectomy and skin regeneration, and other studies all compel us to believe that they exist. Regulator substances are usually discussed in the context of the autoregulation hypothesis, which is composed of the following elements. It is clear that the differentiation of any cell type for performance of a particular, specialized function (for example, hepatocytes) at the same time includes the differentiation of a highly characteristic regulation of the reproductive rate for that given cell type. Quite obviously, differentiation of a fully functional hepatocyte can only be successful if this is accompanied by differentiation of the appropriate rate of cell reproduction (i.e., a rate sufficient to replace liver cells lost by death). If this differentiation of reproductive rate is incomplete or otherwise defective, a hepatoma is the result.

The differentiation of regulation over the rate of cell reproduction also includes a flexibility that allows the cell to accelerate greatly its rate of reproduction in response to the acute loss of
Fig. 2. A scheme for the regulation of reproduction of tissue cells. The scheme encompasses and connects the autoregulation hypothesis and the initiator protein concept. (See text for details). The cell restricts its own reproduction by synthesizing factors (co-repressor and repressor in this figure) that work together to prevent the synthesis of the RNA responsible for production of initiator proteins. The inhibition of the synthesis of initiator proteins prevents the initiation of DNA replication.

other cells of the same type in any renewable tissues (e.g., the increase in reproduction of hepatocytes in response to partial hepatectomy). Therefore, it has been postulated (Fig. 2) that, as a part of differentiation, each particular cell type establishes and maintains a specific microenvironment for itself by continuous addition to the microenvironment of a short-lived co-repressor that specifically interacts, by feedback, to inhibit cell reproduction in that particular cell type which produced the particular co-repressor. The production of the postulated co-repressor not only must be tissue specific but also must be tied in some fashion to the functional demands placed on the particular cell type in order to explain the capacity of renewing tissues to undergo hyperplasia or regression in response to a variable functional demand placed on the tissue. The co-repressor must also be liable or actively broken down if the hypothesis is to explain the dynamic response following such cell losses as partial hepatectomy.

The essence of the hypothesis must certainly be correct, although a regulator substance (co-repressor) has never been identified. What is important here is that
autoregulation, whatever its molecular mechanisms, works by holding cells in the G₁ phase of the cell cycle, and breakdown in a link of the autoregulatory mechanism results in neoplasia by allowing the entrance of cells into DNA synthesis. Without knowing any of the molecular details in autoregulation, we can hardly speculate about the specific details of their breakdown. Nevertheless, there are important indirect clues.

It is well established that a change in chromosomal constitution is a general accompaniment of the development of neoplasia whether the initiating stimulus is chemical, viral, radiation or unidentified (spontaneous). The karyotype changes are usually extensive and variable from neoplasm to neoplasm, and it has not been possible (with one or two exceptions) to associate any regular pattern of change with any particular neoplasm. It has been argued that the gross karyotype changes are secondary to the transformation of a cell to a neoplastic state and that the initial primary change is more subtle. It is, in fact, difficult to understand why gross chromosomal changes would be necessary to produce the G₁ phase breakdown in control of cell reproduction. A human tumor cell line with an apparently normal diploid karyotype has been reported. In a few neoplasms the chromosome change that accompanies transformation is indeed small (e.g., the loss of a small piece of chromosome 21, the Philadelphia chromosome, present in chronic myelogenous leukemia). It seems significant that the loss of part of chromosome 21 in chronic myelogenous leukemia is detectable in normoblasts and megakaryocytes but is only effective in producing neoplasia in leukocytes. The observation is in keeping with the hypothesis that the breakdown in the regulation of cell reproduction is an intimate part of the differentiation of the particular cell type (in this case, leukocytes), and, therefore, transformation of different cell types (in this case, normoblasts and megakaryocytes) requires changes in different parts of the genetic apparatus.

In those cases of chronic myelogenous leukemia that lack any detectable loss from chromosome 21, it can be argued that the crucial deletion underlying this disease occurs at the very end of the chromosome, and that the deletion is therefore accomplished whether a large or undetectably small deletion is involved. The same argument applies to the apparently normal diploid human tumor mentioned in the paragraph above.

This argument on the minuteness of the chromosomal change necessary to produce neoplasia gains validity from the important discovery by Gateff and Schneideman that the development of an invasive, malignant, transplantable neuroblastoma in Drosophila larvae is due directly to the homozygous presence of a recessive mutation in the left tip of the second chromosome (with no other chromosomal change and certainly no change in karyotype). This clear evidence of a single mutational basis for a neoplasm is mindful of the unrecognized work of Gordon many years ago proving beyond doubt the genetic basis of melanoma induction in certain fish. The fact that the neoplastic growth occurs only in neural tissue in Drosophila larvae even though the mutation is present in all tissues, and that the neoplastic growth occurs only in melanocytes in fish even though the same genetic makeup is present in all tissues, strongly indicates that transformation to the cancerous state is connected to a failure of the particular cell type to differentiate the normal regulatory mechanism that is unique to the differentiation process of that cell type. Obviously, it would be important for our understanding of cancer if the activi-
ty specified by the mutant locus in Drosophila could be identified. There appears to be, at present, no feasible way of making such an identification.

The fact that all neoplasms develop by a change in some G1 event focuses special attention on this part of the cell cycle. Of particular importance is the transition from the G1 into the S phase, because the initiation of DNA synthesis is the earliest identifiable point at which it is clear that the cell has become committed to another cell division. The transformation into a neoplastic state is first detected as the acceleration of entrance of cells into DNA synthesis. The breakdown in regulation of the initiation of DNA synthesis is therefore currently the most specific event by which we are able to explain the breakdown of regulation of cell reproduction.

A lesion in the control mechanism for DNA synthesis obviously is the principal issue in the development of neoplasia, but identification of the lesion seems very unlikely before the fully intact control mechanism is elucidated. Most of what is known about the control of DNA synthesis comes from the extensive and ingenious studies of microorganisms. It has been established that initiation of synthesis is brought about by the synthesis of a protein that apparently does not constitute any of the enzymes known to be necessary for DNA synthesis but instead acts as an initiator of DNA synthesis. What controls the synthesis of this initiator protein is one of several key questions. Mutants have been obtained in microorganisms in which the ability to initiate DNA synthesis has been lost, with some evidence that the alteration or loss of the initiator protein is the basis for the defect in initiation of DNA replication. Such information and concepts developed for microorganisms are beginning to have a profound impact on efforts to understand the control of DNA synthesis in higher cells, and it is likely that the development of new approaches to basic research on cancer will be guided more and more by the discoveries from microbial studies.

Figure 2 contains a speculative diagram of a tissue cell that attempts to encompass and connect, with the least number of postulated elements as possible, both the autoregulation idea and the initiator protein hypothesis. Initiation of DNA synthesis is postulated to be brought about by an initiator protein, the synthesis of which is controlled at the transcription level (messenger RNA synthesis). The synthesis of this messenger RNA is repressed by the complex between a repressor protein and a co-repressor. Both the repressor protein and the co-repressor are synthesized continuously, and both are specific for any given cell type; the repressor protein remains intracellular while the labile co-repressor equilibrates with the space of the microenvironment. As long as there is sufficient co-repressor in the system, DNA synthesis is repressed. If the effective concentration of the labile co-repressor in the microenvironment is reduced by acute loss of cells, or is destroyed at an increased rate as a consequence of increased functional demand on the particular cell type, repression of DNA synthesis is weakened in some proportion to the decrease in the amount of repressor-co-repressor complex. The active destruction of co-repressor at a rate dependent on the functional load is, in this scheme, proposed as the means by which functional load is communicated to the regulation of DNA synthesis and cell reproduction.

Without presenting the details of the argument, it seems most likely that the breakdown in the mechanism that controls DNA synthesis most likely would occur in the regulation of synthesis of repressor protein, or in a
mutation of the repressor protein gene itself. The development of the proper amount of regulation may be considered a part of the total pattern of differentiation of that cell type; the less the differentiation of the cell, the less complete the development of regulation of repressor synthesis. Inclusion of the property of specificity by cell type for both co-repressor and repressor is sufficient to explain independent regulation from one tissue cell type to the next and is particularly pertinent to the fact that neoplasia are highly tissue specific.

All these hypothetical arrangements may yield a picture that seems just too contrived and fanciful in view of the dearth of solid information, but most of this scheme has a counterpart in the mechanism of regulation of RNA transcription in microorganisms that is based on firm fact. Elements of the diagram (Fig. 2) have already been published by others.

In any case, the elucidation of the molecular mechanisms that control the initiation of DNA synthesis will put within much closer reach a full explanation of the regulation and the loss of regulation of cell reproduction in mammalian tissues. At this point it is quite clear that the problem of loss of regulation of cell reproduction in neoplasia is precisely a question of a genetically based loss of control over initiation of DNA replication, and until we solve these pointed problems, the chance of finding a prevention or cure to cancer is extremely remote.

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