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Review

The Role of Stem Cells and Gap Junctional Intercellular Communication in Carcinogenesis

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Understanding the process of carcinogenesis will involve both the accumulation of many scientific facts derived from molecular, biochemical, cellular, physiological, whole animal experiments and epidemiological studies, as well as from conceptual understanding as to how to order and integrate those facts. From decades of cancer research, a number of the "hallmarks of cancer" have been identified, as well as their attendant concepts, including oncogenes, tumor suppressor genes, cell cycle biochemistry, hypotheses of metastasis, angiogenesis, etc. While all these "hallmarks" are well known, two important concepts, with their associated scientific observations, have been generally ignored by many in the cancer research field. The objective of the short review is to highlight the concept of the role of human adult pluri-potent stem cells as "target cells" for the carcinogenic process and the concept of the role of gap junctional intercellular communication in the multi-stage, multi-mechanism process of carcinogenesis. With these two concepts, an attempt has been made to integrate the other well-known concepts, such as the multi-stage, multi-mechanisn or the "initiation/promotion/progression" hypothesis; the stem cell theory of carcinogenesis; the oncogene/tumor suppression theory and the mutation/ epigenetic theories of carcinogenesis. This new "integrative" theory tries to explain the well-known "hallmarks" of cancers, including the observation that cancer cells lack either heterologous or homologous gap junctional intercellular communication whereas normal human adult stem cells do not have expressed or functional gap junctional intercellular communication. On the other hand, their normal differentiated, non-stem cell derivatives do express connexins and express gap junctional intercellular communication during their differentiation. Examination of the roles of chemical tumor promoters, oncogenes, connexin knock-out mice and roles of genetically-engineered tumor and normal cells with connexin and anti-sense connexin genes, respectively, seems to provide evidence which is consistent with the roles of both stem cells and gap junctional communication playing a major role in carcinogenesis. The integrative hypothesis provides new strategies for chemoprevention and chemotherapy which focuses on modulating connexin gene expression or gap junctional intercellular communication in the premalignant and malignant cells, respectively.

Keywords: Carcinogenesis, Chemoprevention, Gap junctional intercellular communication, Epigenetic, Stem cell

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"Those researching the cancer problem will be practicing a dramatically different type of science than we have experienced over the past 25 years. Surely much of this change will be apparent at the technical level. But ultimately, the more fundamental change will be conceptual" (Hanahan and Weinberg, 2000).

Introduction

This quotation by Hanahan and Weinberg, from their paper on the " Hallmarks of Cancer", highlights the major objective of this mini-review of a very complex and controversial area of research, namely, that, even though great discoveries of both conceptual and technical nature have been made, a major "gap" of knowledge needs to be gained before we can thoroughly explain carcinogenesis. Their claim is that in order for real advances in our goal to understand, hence to prevent and to treat cancers better than we are currently doing, we must find new concepts to organize the plethora of experimental findings. All of the pioneering work done by

molecular oncologists, biochemists, virologists, pathologists, geneticists, cell biologists, epidemiologists, etc. provides the foundation which Newton described: "I saw further because I stood on the shoulder of giants". Summarizing some of the general features of all cancers forged by these "giants", they listed: (a) the acquisition of independence from growth signals; (b) the ability to become insensitive to growth inhibitory mechanisms; (c) ability to escape the apoptotic machinery; (d) to develop the ability to have unlimited replication potential; (e) the generation of its own angiogenesis support system; and (f) having the properties of invasiveness and metastasis of tissues and organs (Hanahan and Weinberg, 2000; Boettner and Van Aelst, 2002).

To generate new concepts in order to forge a framework to give meaning to all the information being produced from the different fields of cancer research, I will hypothesize that two more concepts, namely the "stem cell theory of carcinogenesis" and "the gap junctional intercellular communication" concept, can help to integrate many of the previously speculated theories and observations (Trosko *et al*., 1993; Trosko and Ruch, 1998; Trosko and Chang, 2001; Trosko and Ruch, 2002). While neither concept is new, what is new is that they both appear to be related to each other, as well as to the carcinogenic process (Trosko and Ruch, 1998). With the isolation of adult stem cells and the demonstration that these adult stem cells do not express connexin genes or have functional GJIC until they are induced to differentiate, provide the foundation of the hypothesis of this mini review, namely, the target cells for carcinogenesis are the adult stem cells, which are constitutively "immortal" until induced to express connexin genes and to differentiate (Chang *et al*., 1987; Kao *et al*., 1995; Matic *et al*., 1997; Sun *et al*., 1999; Dowling-Warriner and Trosko, 2000; Matic *et al*., 2002). If these stem cells or their very early differentiated daughter cells, that have started to express their connexins and to differentiate but not yet down- regulated their telomerase activity, are exposed to a carcinogenic "initiator" (see section, "Integrated Hypothesis of Carcinogenesis"), they will remain "immortal". If expanded by mitogenic means and/or prevented from apoptotic death, they can accrue addition genetic and epigenetic changes to acquire these "Hallmarks of Cancer".

What has been generally ignored in the cancer field is the early and original observation of Loewenstein (Loewenstein, 1966) that gap junctional intercellular communication is associated with growth control and differentiation in normal cells and that cancer cells, characterized by the lack of growth control and the inability to terminally differentiation, do not appear to have either functional homologous or heterologous GJIC (Yamasaki *et al*., 1987). In addition, about that same time, Eagle (Eagle, 1965) noted, "contact inhibition" was a property of normal cells. Moreover, Borek and Sachs showed that this property of "contact inhibition", which was lost in cancer cells, was associated with the absence of functional GJIC (Borek and Sachs, 1966).

Concepts of Carcinogenesis

Both animal carcinogenesis and human epidemiological studies, as well as molecular and in vitro studies, have clearly demonstrated one fact about the formation of a cancer, namely, it is a multi-stage, multi-mechanism process (Weinstein *et al*., 1984**)**. The concept of "Initiation/Promotion/ Progression" (Pitot and Dragan, 1991) implies that, when a single normal cell is exposed to an agent that can irreversibly alter a gene, such that it can no longer terminally differentiate, it will remain immortal and has been "initiated" (Trosko and Ruch, 1998). The initiated cell is not a tumor cell but it has incurred the first step on the way to become a tumor after many more genotypic/phenotypic changes have been induced in that cell. The initiation process inhibits the ability of the target stem cell to divide asymmetrically, allowing only symmetrical cell division. This means every time an initiated cell is stimulated to divide, it only can produce two like-type initiated cells. It is unable to divide asymmetrically to produce a terminally-differentiated daughter and one initiated stem cell. If that cell remains suppressed by surrounding cells, no tumor will appear. If, on the other hand, agents which inhibit the suppressing effects of the surrounding normal cells, this initiated cell, which will not terminally differentiate, will proliferate into a mass of non-terminally-differentiated cells in a tissue (Trosko *et al*., 1996). This is the "promotion" phase of the carcinogenic process (Trosko, 2001). The nodule of the breast, the papilloma in the skin, the polyp of the colon, and the enzyme- altered foci of the liver are all examples of a single initiated cell that was multiplied by promoters into a mono-clonally-derived, partially differentiated mass of cells. Finally, if one of those promoted, initiated cells accrues additional genetic or "epigenetic" changes such that it can invade and metastasize to other tissues, the conversion to the "progression" or malignant stage of carcinogenesis has occurred (Pitot *et al*., 1981).

The stem cell theory (Trosko and Chang, 1989) is another concept that helps to integrate the observations related to the initiation/promotion/progression theory. "Oncogeny as partially blocked ontogeny" theory was proposed by Potter (Potter, 1978) to explain the observation that cancer cells can range in phenotype from being very "embryonic- like" to being almost terminally differentiated. However, they are all mono-clonally-derived from a stem or stem-like cell (Fialkow, 1979). These stem cells, which by definition, are immortal until they are induced to become terminally differentiated or "mortalized". Once these stem cells are prevented from terminally differentiation, they are "initiated". If these initiated stem cells are stimulated to proliferate, they cannot terminally differentiate, as do normal stem cells. Therefore, they accumulate in the tissue as abnormal clones of non-terminally-differentiated tissue.

The oncogene / tumor suppressor gene theory of carcinogenesis (Weinberg, 1991) now can be integrated into the stem cell theory and the initiation- promotion-progression

theory. While we know there are over a hundred oncogenes which code for a number of different functional proteins (growth factors, receptors, signal transducing agents and transcription factors) and a growing number of tumor suppressor genes, we do not know which of these genes might be that which is altered during the "initiation" stage of cancer cell. However, its function must be to prevent the terminal differentiation of a stem cell and to prevent it from "mortalizing". Since the "initiated" cell can be suppressed from uncontrolled cell proliferation by surrounding normal cells, the initiation step probably does not involve a tumor suppressor gene because that is the function of this class of genes. There are two types of tumor suppressor genes, namely those that code for (a) negative growth factors / negative growth factor receptors; and (b) gap junctions (protein structures in membranes of cells which are channels to provide direct transfer of ions and small molecules directly between the cytoplasms of neighboring cells).

Mutation / epigenetic theories (Trosko and Chang, 1988) of carcinogenesis helps to integrate "initiators", that are agents causing irreversible changes in the genes of the stem cell, with the "promoters", which are agents that can either stimulate the growth of the initiated cells and/or block the death of these initiated cells (apoptosis) in an interruptible or even reversible fashion. Mutations can therefore explain "initiation" because it is an irreversible process [Although not all "initiation" events need to be caused by mutagenic events. Some might be the result of stable "epigenetic" mechanisms at the transcriptional level, such as that which might occur in teratomas.]. Promotion is an epigenetic process (altered gene expression at the transcriptional, translational or posttranslation levels) that can be a reversible process. It does not alter the genetic information, but only the expression of that information.

Integrative -ypothesis of Carcinogenesis

Finally, the role of gap junction intercellular communication (GJIC) in the new integrative hypothesis of carcinogenesis can now bring together each of the preceding hypotheses. Stem cells are suppressed from growth by negative growth regulators and negative growth receptors. They do not appear to have gap junctions (Trosko *et al*., 2000). If induced to start differentiating, gap junctions are needed. However, if the stem cell has been initiated, they can start to differentiate. Since one of the critical oncogenes as been irreversibly altered or mutated, they cannot go all the way to the terminally differentiated stage. Because they have gap junctions, they can partially differentiate and can be "contact-inhibited" or suppressed from uncontrolled cell proliferation. If the GJIC is reversibly inhibited by mitogenic factors (tumor promoters) (Trosko and Chang, 2001) and/or prevented from apoptosing (Lee, 2000), the GJIC-mediated contact inhibition is suppressed and these cells can proliferate but not terminally differentiate. After a long period of stimulation of growth by either endogenous mitogens (e.g., growth factors or hormones) or exogenous factors (environmental or dietary agents), additional changes could occur in one of these amplified initiated, immortal stem cells. If that change involves a stable down regulation of GJIC by an endogenous gene, no longer does the benign tumor need an exogenous tumor promoter to stimulate growth. Growth can be stably stimulated by an activated oncogene together with the inactivated tumor suppressor gene.

Mechanistic Based Strategy for Chemoprention and Chemotherapy

The current approach to treat cancers is obviously bankrupt. It is simply based on trying to kill or physically remove the cancer cells in the patient with radiation and/or toxic chemicals or by surgery before the treatment kills the patient. Based on simplistic views of current reductionalistic knowledge of carcinogenesis, multiple strategies for cancer treatment have been proposed: inhibitors to cell cycle enzymes; inducers of apoptosis or differentiation; inhibitors to telomerase; targeted antibodies to cancer "antigens"; gene therapy; angiogenesis inhibitors; bystander treatment; enhancers of immune system; and oncogene product inhibitors. However, all these have viewed the cancer cell independent of the context of the whole organism. Specifically, few, if any, of these approaches have been based on the integration of all of the concepts regarding the carcinogenic process.

To begin, not all cancers are alike, except for one apparent phenotype, namely, cancers, derived from solid tissues, are unable to perform gap junctional intercellular communication. Dysfunctional GJIC could be the result of (a) the gene for the connexins not being expressed (e.g., HeLa or MCF-7 cancer cells) or (b) the connexin proteins and gap junctions not being translated, transported, assembled or being functional due to a mutation, abnormal splicing of message or being modified by oncogenes or chemical tumor promoters. Cancers from soft tissues, which do not need GJIC, must, however, need to communicate via negative growth regulators; therefore, they must have functional receptors for these negative growth suppressors (such as TGF-beta). If these negative growth receptors (or their down stream effector signaling components) are not expressed, or are mutated, the cells cannot differentiate or be growth controlled.

Based on an observation by Lee (Lee, 2000), that there seems to be two kinds of tumors in any organ (i.e., estrogen receptor positive and negative breast cancers), a hypothesis has emerged that has modified the original stem cell theory (Trosko and Chang, 1989). First, the two major theories of the target cell for carcinogenesis are the pluripotent stem cell or the de-differentiated cell (Sell, 1993). For the sake of brevity, it will be assumed that the stem cell theory is the correct

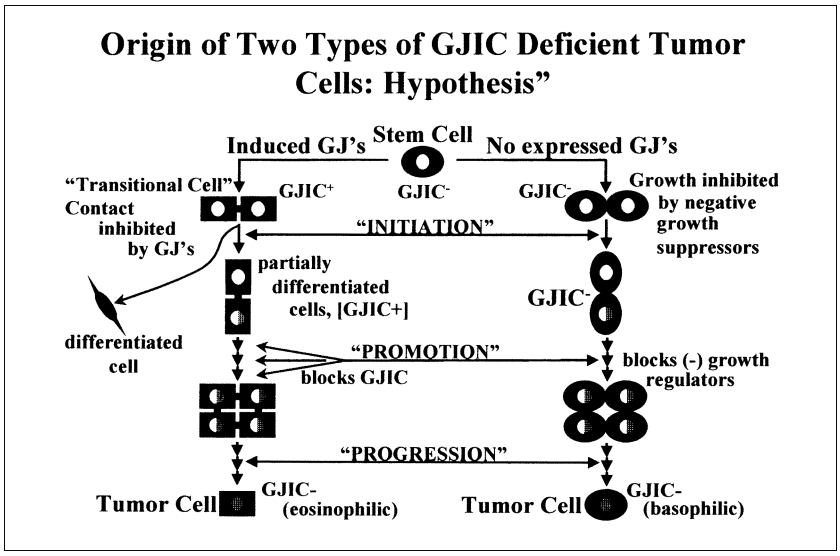

Fig. 1. This diagram illustrates how two types of cancer cells could arise from either pluri-potent stem cells (lacking expressed connexin genes and having no GJIC) or from very early transit cells, which express connexin genes and have functional GJIC after exposure to an initiator. Initiation is that process which would prevent the stem or transit cell from terminal differentiation [loss of telomerase activity]. These initiated stem or initiated transit cells would be growth suppressed either by secreted negative growth regulators or by gap junction-dependent "contact inhibition", respectively. If these initiated stem or initiated transit cells are exposed, chronically, to agents that either inhibit the secreted negative growth regulator or its receptor-dependent signaling (initiated stem cell) or down regulate gap junctional intercellular communication (initiated transit cell), these initiated cells would proliferate, accumulate and accrue sufficient genetic/epigenetic changes sufficient to become "promotor independent" and invasive and metastatic. In the end, both tumor types lack function GJIC, one due to the transcriptional suppression of the connexin genes [stem cells]; the other because various mutations/activated oncogenes/de-activated/loss of tumor suppressor genes cause down regulation of the expressed connexins and gap unctions [transit cells]. Strategically and tactically, based on this hypothesis, the approach to chemoprevention and chemotherapy would be very different.

theory. However, in view of the observations by Lee (Lee, 2000), the target cells could be the pluripotent stem cell of all adult tissues, but also, the early "transit" daughter cell of the stem cell that has been induced to start differentiation by having its connexin genes expressed and made functional. The pluripotent stem cell would have negative growth receptors to regulate its growth and differentiation. If "initiated", these stem cells might not be able to terminally differentiate but can still be growth controlled as long as the negative growth factor and the negative growth factor receptor are present and functional and the down stream effector signaling is functional. If these initiated soft tissue stem cells are multiplied to sufficient numbers by mitogens or inhibitors to the negative growth factors, such that additional alterations occur to the genes regulating the negative growth regulators and/or down stream effectors, these become the un-terminally differentiated, un-growth controlled soft tissue tumors. Promoters for these types of initiated cells would be factors that inhibit the negative growth factor from suppressing growth, and that would stimulate mitogenesis and prevent apoptosis.

On the other hand, the "target" cells from solid tumors would not only contain the pluri-potent stem cells, which, also do not express connexins or have functional GJIC (Trosko and Ruch, 1998), but which are growth suppressed by negative growth factors. In addition, the solid tissue would contain some early progenitor or stem like cells, which do have expressed connexins and functional GJIC. These cells have not yet lost their telomerase activity and are not yet committed to terminally differentiate or to senesce. As in the previous case, the pluripotent stem cell without GJIC could become initiated, promoted by factors that reduce the negative growth factors or which down - regulate the receptors and down stream effects of the negative growth factor receptor. However, the early progenitor cell, that still has stem - like potential, has GJIC. Therefore, if initiated, these cells are unable to terminally differentiate but they are still growth controlled by functional GJIC. These initiated cells in solid tissues can be promoted by agents that down regulate GJIC. This causes these initiated cells to escape growth control and they do not apoptosis (Trosko and Ruch, 1998). Upon expansion of these initiated cells into a large mass (i.e., polyps in the colon), addition alterations can occur such that GJIC is now down-regulated stably by endogenous means (activated oncogenes which could alter phosphorylation of the connexin proteins). These cells now become autonomously able to grow without the need of exogenous tumor promoters. Eventually, they acquire other changes need for invasiveness and metastasis.

In summary, the stem cell derived tumor would be very "embryonic-like" (basinophilic), while the early progenitor

stem cell would start to have some differentiated features, including GJIC (eosinophilic). Promoters of the former would need to ameliorate the negative growth factor suppression of the initiated cell to cause expansion of these blocked differentiated cells. These cells would probably not have activated oncogenes or in-activated tumor suppressor genes. Promoters of the latter type would need to down regulate, reversibly, GJIC either by exogenous factors (chemical tumor promoters or hormones, growth factors) or by activated oncogenes or in-activated tumor suppressor genes that stably down regulate the function of GJIC. These tumors would have expressed oncogenes and/or de-activated tumor suppressor genes, as well as the expressed but non-functional connexins.

Consequently, not only must we recognize that each tumor, not only within an organ but also between organs, is different from each other, but also that there will be two basic types within each solid tissue, namely, those that express their connexins (eosinophilic) and those that do not (basophilic). Possibly, for example, estrogen receptor - negative and estrogen receptor positive breast cancers might be examples of tumors with suppressed connexin gene type tumors and connexin protein modified type tumors, respectively. In addition, the basal cell skin carcinoma and squamous cell carcinomas might also be a reflection of this hypothesis. Therefore, the major implications of all these observations are that (a) there will never be a "golden bullet" to treat all cancers alike; (b) treatment of tumors with expressed connexins, activated specific oncogenes and inactivated tumor suppressor genes will not only have to be targeted to the specific oncogene / tumor suppressor gene in that tumor, but will be different from those tumors without activated oncogenes / inactivated tumor suppressor genes and with no expressed connexins [Treatment of HeLa or MCF-7 like tumors will have to be very different than tumors having a mutated Ha - Ras oncogene and mutated p53 tumor suppressor genes.]; and (c) more emphasis will have to be placed on prevention of cancers rather than on treatment.

Chemoprevention: a Strategy to Prevent the Expansion of Initiated Cells to Become Malignant

Lets be realistic, while we can prevent some initiation of our cells, we can never completely eliminate spontaneous or uncontrollable initiation of some of our stem cells. However, since the tumor promotion phase of carcinogenesis must occur in a regular, sustained fashion over a long period of time to allow the clonal expansion of the single initiated cell to escape apoptosis and to accrue the changes needed for that initiated cell to become autonomous of exogenous growth stimulators and to acquire invasive and metastatic properties, this step is the most logical for chemo-prevention strategies. If the mechanism of tumor promotion involves the down regulation of GJIC, which controls cell proliferation, apoptosis and terminal differentiation, chemo-prevention must interfere with the promoters effect on GJIC (Trosko and Ruch, 2002). Chemotherapy, on the other hand, must deal differently with either the tumor cell with no expressed connexins and no GJIC or with the tumor cells with expressed connexins and no GJIC.

Many dietary and life style intervention means can be utilized. Caloric restriction in animals seems to be a significant means to reduce all chronic diseases, including cancer. In other words, while it would not be realistic to calorically - restrict our diet so as to be painful (starvation diets), we can do much to reduce being overweight and obese. In addition, simply by reducing calories is not wise without knowledge of nutrient requirements for not only general health (We must have essential fats, minerals, vitamins, etc), but the diet must contain foods that contain factors that can either increase GJIC or prevent the down regulation of GJIC by tumor promoters.

The bottom line is that we must incorporate the concept that healthy cells must communicate with each other. We can do much to prevent our cells from not communicating by both our life style and diet. Since, even with our best intentions, cancers will always appear in humans, we must approach cancer therapy with this same concept that cancer cells are cancerous because they do not communicate with their normal counterparts. Therefore, the strategy will be to recognize why they do not communicate (Are the connexin genes expressed or are they expressed but not functional?). Dr. Van R. Potter had it correct when he stated: "The biochemistry of cancer is a problem that obligates the investigator (or clinician) to combine the reductionalistic approaches of the molecular biologists with the holistic requirements of hierarchies within the organism. The cancer problem is not merely a cell problem; it is a problem of cell interaction, not only within tissues, but also with distal cells in other tissues. But in stressing the whole organism, we must remember that the integration of normal cells with the welfare of the whole organism is brought about entirely by molecular messages acting on molecular receptors" (Potter, 1978).

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