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Gap Junctional Intercellular Communication: A "Biological Rosetta Stone" Concept for Understanding Epigenetic Toxicology

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***ABSTRACT**: Some would argue that the search for the origin and treatment of this disease will continue over the next quarter century in much the same manner as it has in the recent past, by adding further layers of complexity to a scientific literature that is already complex almost beyond measure. But we anticipate otherwise: 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).*

I. INTRODUCTION

This quotation from Hanahan and Weinberg will serve as the foundation of the working hypothesis of this brief but provocative review. Clearly, they have highlighted the limitations of the reductionalistic successes of molecular and cellular biology. With the sequence of the human genome at our finger tips, the identification of hundreds of proto-oncogenes, tumor suppressor genes, scores of human syndromes inheriting cancer and other predisposing chronic diseases, and with the availability of scores of incredibly sensitive technologies, we are still no where near understanding cancer and other chronic diseases so that we have adequate control of their prevention or treatment. Indeed, as Hanahan and Weinberg point out, even more information will be generated via this approach. Yet more information is neither understanding nor wisdom. What is truly needed is a new organizing paradigm or conceptual framework. Diseases are not mutated genes or biologically-abnormal cells alone. Potter has clearly articulated this point when outlining the complexity of the cancer problem: "The biochemistry of cancer is a problem that obligates the investigator to combine the reductionalistic approaches of molecular biologist with the holistic requirements of hierarchies within the organisms. 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 also remember that the integration of normal cells with the welfare of the whole organism is brought about entirely by molecular messengers acting on molecular receptors" (Potter, 1973).

A disease is really a disrupted homeostatic system in a biological organism consisting of a hierarchy of cybernetically-interacting systems [atomic/molecular/cellular/tissue/organ/organ systems] (Brody, 1973; Potter, 1974). Disease will have to be viewed as a "complex system" (Weng *et al.*, 1999).

In the past, from a reductionalistic point of view, it seemed both logical and practical to treat birth defects, cancers, cataracts, immune disorders, reproductive and neurological/mental dysfunctions as unique and distinct disease entities for it seems obvious they are clinically-distinct. Yet, it had not gone unnoticed by some that many of these diseases seemed to share common etiological factors (e.g., genes, diet, life styles, pollutant or workplace factors) and, in several cases, multiple diseases showed up quite commonly in the same individual either with inherited predisposing genes or exposure to an environmental chemical. Birth defects commonly appeared in children with cancers (Miller, 1977; Trosko and Chang, 1984), atherosclerosis shared many pathogenic similarities with carcino-

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genesis, as well as with etiological factors (Majesky *et al.*, 1985). Chemicals that caused tumor promotion, also seemed to modulate the immune system or effect (Rosenkranz *et al.*, 2000).

Therefore, the radical idea to be proposed is that diseases, such as a birth defect, a cancer, a reproductively sterile adult, a immune altered individual, a person with a number of neurological or mental disorders, or with cataracts, share a common underlying mechanism, namely the disruption of homeostatic control of cell proliferation, cell differentiation, apoptosis and of adaptive responses in the differentiated cells.

II. EVOLUTIONARY BASIS OF HOMEOSTATIC CONTROL IN MULTICELLULAR ORGANISMS

Bacteria survived and evolved by having rare mutations in a large population that conferred some selective advantage when the environment changed that caused the prevailing set of genes inadequate to cope with the changes. They basically survived by cell proliferation which was controlled fundamentally by the external constraints of nutrient availability and temperature. With the appearance of the first multicellular organism came several new phenotypes, namely (a) internal growth control; (b) cellular differentiation; (c) apoptosis; (d) adaptive responses of the terminally differentiated cells and (e) senescence. In addition, the development of "totipotent stem" cells (cells capable of giving rise to all cell types in the multicellular organism), pluripotent stem cell (cells restricted in the types of cells to which it can give rise), committed progenitor cells (cells that give rise to a limited number of cell divisions and to a specific differentiated type) and the terminally differentiated cell (cells incapable of any further cell division). These concepts assume, of course, the normal developmental microenvironment for these definitions to hold. With recent observations of transdifferentiation or de-differentiation of stem cells or even differentiated somatic cells, these definitions are bound to change (Lake, *et al.*, 2000; Lanza *et al.*, 2000; Jackson *et al.*, 1999; Clarke *et al.*, 2000).

Consequently, the appearance of the first multicellular organisms required a new biological process to orchestrate (a) the development of an organism such as a human being with 100 trillion cells from a single fertilized egg; and (b) the simultaneous multiplication of cells with the differentiation of other cells, while certain cells died by apoptosis and others started doing adaptive differentiated responses. All of these very different cellular processes had to occur errorlusly during embryogenesis, fetal development, sexual maturation and during adulthood in order not to upset any of the cellular processes and the structure/function of the organism. This process to maintain control of these cellular/tissue/organ/organ system structures/functions is called homeostasis.

Homeostasis (or the tendency to stability in a multicellular organism that is achieved by a system of control mechanisms activated by a balance of positive and negative feedback of molecular information.), while it exists within single cells, refers to the control of cell behavior within/between other tissues/organs. This idea has been missing in modern reductionalistic molecular biology yet it is necessary to understand that the higher order phenomena that emerges from the organization and interaction of the hierarchical levels (molecular/biochemical/cellular/tissue/organ/organ systems) is what constitutes the "complex system" (Trosko *et al.*, 1998) of what a human being is. A human being is not "just" a collection of 100 trillion cells, but a homeostatically interaction between stem, progenitor and differentiated cells with the intrinsic micro-environment (extracellular matrices) and extrinsic macro-environment (physical, dietary, drug, and chemical). To help maintain this delicate homeostatic control, a series of communication mechanisms evolved in an integrated manner.

Conceptually, homeostasis can be seen as the integration of "extra-, intra- and inter- cellular communication (Fig. 1). Cells can communicate via molecules (hormones, growth factors, cytokines, neurotransmitters) excreted by one cell type that effects cells at a distal site [extra-cellular communication]. Once the target cell has received the extra-cellular signal, various kinds of "intra-cellular" signals (activation of protein kinases, transcription factors, ion channels, increases in intracellular Ca++ or c-AMP, etc.) then modulates gap junctional intercellular communication (GJIC) by either increasing or decreasing the gap junction function, as well as modulating gene expres-

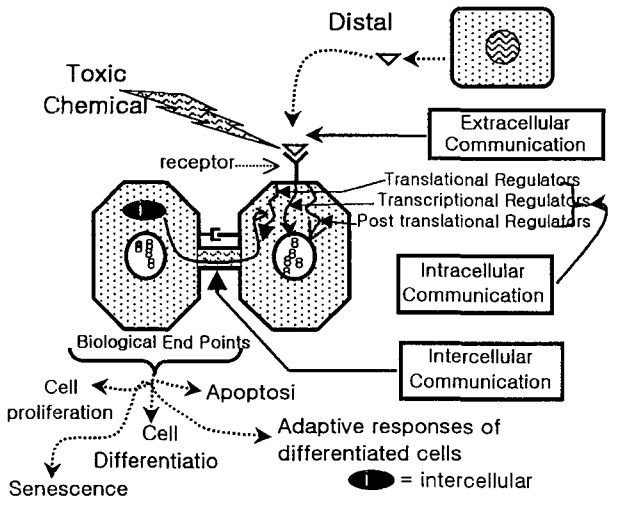

Fig. 1. Endogenous and exogenous extra-cellular signals which can trigger various intra-cellular signal transduction mechanisms can either increase or decrease gap junctional inter-cellular communication between cells in a multicellular organism. Cell proliferation, differentiation, apoptosis and adaptive responses of differentiated cells can occur as a consequence of the modulation of GJIC. (From Trosko *et al.*, *Toxicology Letters*, 102-103: 71-78, 1998; with permission from Elsevier Science)

sion. Since most cells are also "anchored" to particular extracellular substrates and attached to each other by cell adhesion molecules in addition to their tight and gap junctions, the net effect of all these signals (extra, GJIC-mediated, extra-cellular matrix-mediated and cell-mediated-mediated) determine the fate of the cells response [no response or G0; cell proliferation; cell differentiation; apoptosis; or adaptive response in the terminally differentiated cell].

III. GAP JUNCTIONAL INTERCELLULAR COMMUNICATION: THE INTEGRATOR FOR MULTICELLULAR HOMEOSTASIS

The key to this concept is the introduction of the genes for the gap junction structure ("connexins"). The gap junctions are structures not found in single cell organisms. They appeared during evolution when the metazoan appeared (Revel, 1988) therefore, their appearance occurred at the time when the new phenotypes of multicellular organisms appeared (e.g., growth control, differentiation, apoptosis, adaptive responses of differentiated cells). The fundamental question is: "Are these phenotypes causally or coincidentally related to the appearance/function of gap junctions?"

Gap junctions are channels, consisting of two joined hemi-channels ("connexons") in the membranes of contiguous cells, that are composed of proteins (connexins) organized into the heximeric connexons (Bruzzone *et al.*, 1996). Ions and small molecules can pass through the gap junctions (Loewenstein, 1979) to assist in either electrotonic or metabolic synchronization of excitable and non-excitable cells, respectively (Finbow and Yancey, 1981). Both homologous and specific heterologous cell coupling can and does occur (Brink *et al.*, 1997). GJIC can be modulated (increased or decreased), transcriptionally or posttranscriptionally, by both endogenous factors [hormones, growth factors) or by exogenous factors (drugs, dietary factors, pollutants, toxins, etc.] (Trosko and Ruch, 1998).

The roles of gap junctions in growth control (Loewenstein, 1990; Yamasaki and Naus, 1996), differentiation (Lucke *et al.*, 1999), development (Lo, 1996), apoptosis (Wilson, *et al.*, 2000), and the adaptive responses of differentiated cells such as insulin release in pancreatic beta cells (Meda *et al.*, 1987), has been postulated. Moreover, to integrate the concept of stem cells into the picture being developed, it has been known that up until the early blastula stage of an embryo, the individual cells at this stage do not have functional GJIC (Lo, 1996). In addition, several pluripotent stem cells (e.g., human kidney and human breast epithelia, as well as human neuronal, stem cells) do not have expressed GJIC (Chang *et al.*, 1987; Kao *et al.*, 1995; Dowling-Warriner and Trosko, 2000). After induction of GJIC by agents that increase c-AMP, these cells have functional GJIC and start to differentiate.

1. Cancer as a "Disease of Homeostasis"

The universal characteristics of all cancers is their "loss of growth control" and inability to terminally differentiate, as well as abnormal apoptosis. Cancer has been described as a "disease of differentiation" (Markert, 1968), "oncogeny as partially blocked ontogeny" (Potter, 1978) or a "stem cell disease" (Nowell, 1979; Till, 1982). Normal cells "contact inhibit" their growth (Levine *et al.*, 1965), while cancer cells have lost "contact inhibition" (Borek and Sachs, 1966). Interestingly, cancer cells do not have functional homolo-

gous or heterologous GJIC (Yamasaki *et al.*, 1987). Tumor promoting chemicals and a number of oncogenes (ras, src, raf, mos, neu, Bcl-2) can down regulate GJIC, while several tumor suppressor genes can up regulate GJIC (Trosko *et al.*, 1993). Anti-tumor promoting drugs (lovastatin) and chemopreventive chemicals (retinoids, carotenoids, green tea components, caffeic acid phenylether ester) can either up regulate GJIC in cancer cells or prevent the down regulation of GJIC by tumor promoters and oncogenes (Trosko *et al.*, 1998). Strategies to either genetically-engineer non-GJIC cancer cells to increase their ability to have functional GJIC via connexin transfection has restored normal cell growth and the loss of tumorigenicity (Trosko and Ruch, 1998; Zhu *et al.*, 1991; Eghbali *et al.*, 1991; Hirschi *et al.*, 1996; Mehta *et al.*, 1991). One form of the "bystander effect" has been associated with the increase in GJIC (Duflot *et al.*, 1998; Mesnil *et al.*, 1997; Estin *et al.*, 1999; Touraine *et al.*, 1998; McMasters *et al.*, 1998; Wygoda *et al.*, 1997). The generation of a connexin32 knock-out mouse has shown that this connexin, which is normally expressed in the liver, seems not to be required for either the development of a liver or the survival of the mouse, although it predisposes the mouse to a higher spontaneous and chemically-induced liver cancer frequencies (Temme *et al.*, 1997).

GJIC has been postulated to play a role in the multi-stage, multi-mechanism process of carcinogenesis, consisting of the "initiation/promotion/progress" phases (Trosko *et al.*, 1983). Specifically, the reversible and threshold-dependent down regulation of GJIC by non-genotoxic tumor promoting chemicals was hypothesis to be the cellular mechanism of tumor promotion (Trosko and Chang, 1988). Tumor promoters also have been shown to block apoptosis during the tumor promotion phase (Bursch *et al.*, 1992), speculating the idea that GJIC was also necessary for some forms of apoptosis in solid tissues (Trosko and Goodman, 1994). When GJIC is irreversibly down regulated by overexpressed oncogenes, then GJIC plays a prominent role during the progression phase [i.e., invasion and metastasis] (Nicolson *et al.*, 1988).

All of these independent observations, while in isolation, seems, at best coincidental. However, in aggregate, the weight of the evidence, strongly supports the hypothesis that GJIC is a necessary, if not sufficient, process needed for homeostatic control of cell function and its loss can lead to loss of growth control, differentiation and apoptosis.

How do stem cells come into this picture? The prevailing idea of cancer is that, starting with a normal, mortal cell, the carcinogenic process starts by "immortalizes" the normal cell. This immortal cell can, because of its long life span can accrue more mutational/epigenetic events to acquire the malignant, invasive and metastatic phenotype. Some have speculated the normal cell with limited or no activated telomerase activity must be induced to have activated telomerase for immortalization and as it becomes malignant, it increases its telomerase activity (Bodnar *et al.*, 1998). The alternative hypothesis is that the stem cell is naturally "immortal" and only becomes "mortal" when it is induced to terminally differentiate (Trosko *et al.*, 2000). We have shown that normal human breast epithelial stem cells have telomerase activity (Sun *et al.*, 1999).

Therefore, the integrated concept of the multi-stage, multi-mechanism process of the initiation/promotion/progression model of carcinogenesis could be visualized in Fig. 2. Starting with a pluripotent stem cell which can divide asymmetrically to form one differentiated daughter and one stem cell, after exposure to an initiator, the stem cell loses its ability to terminally differentiate, maintains its telomerase activity and losses its ability to divide asymmetrically but divides symmetrically. While the initiation process has not completely blocked differentiation ("oncogeny as partially-blocked ontogeny"), it seems that the partially-differentiated cell can be suppressed by GJIC from its surrounding normal cells. However, after exposure to agents that reversibly inhibit GJIC (tumor promoters), these initiated cells divide symmetrically to increase their numbers because they do not terminally differentiate and they do not die by apoptosis. The enzyme altered foci of rat livers, the papilloma of mouse skin, the nodes in the mammary tissue and the polyps in the colon would be examples of these partially differentiated, monoclonally-derived cells. Withdrawal of the promoting chemicals at stage were GJIC is still reversible would lead to the regression of these benign tumors, probably by apoptosis (Hikita *et al.*, 1999). If the GJIC is stably down regulated by

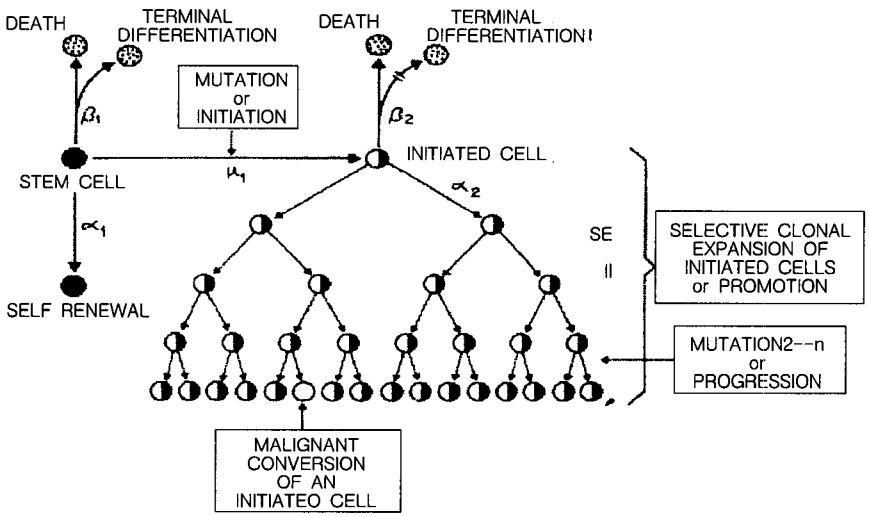

Fig. 2. The initiation/promotion/progression model of carcinogenesis. β_1 = rate of terminal differentiation and death of stem cell; β_2 = rate of death, but not of terminal differentiation of the initiated cell (—| |►); α_1 = rate of cell division of stem cells; α_2 = rate of cell division of initiated cells; μ_1 = rate of the molecular event leading to initiation (i.e., possibly mutation); μ_2 = rate at which second event occurs within an initiated cell. (From Trosko *et al.*, In: Modern Cell Biology; Vol. 7, *Gap Junctions*, E.L. Hertzberg and R.G. Johnson, eds., pp. 435-448, 1998; with permission from Alan R. Liss, Inc., New York).

overexpressed oncogenes, then the cells become tumor promoter-independent and are on their way to become malignant.

IV. MODULATED GJIC IN OTHER DISEASE STATES

Since gap junctions can be composed of over a dozen connexin proteins coded by a highly evolutionary-conserved set of genes (Bennett *et al.*, 1995) and are differentially expressed in various cells/tissues/organs (White and Paul, 1999), and are expressed in all tissues of the body, they obviously are there for a very fundamental and basic reason ["Nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1973)]. They are there to maintain homeostasis or regulation of cell growth, differentiation, apoptosis and adaptive functions of differentiated cells. Therefore the abnormal modulation of GJIC in any tissue at some stage of their development, maturation and adaptive state could lead to a dysfunction of development or function.

The creation of a series of connexin knockout mice has shown that they can be either vital to life (K.O. Cx43 dies in utero) or they influence development and function (K.O. Cx37 leads to female sterility; K.O. Cx32 to predisposing liver tumors). In addition, abnormal GJIC has been associated with dermatological processes, ophthalmological and ontological processes, radiation induced pulmonary injury, hepatic and gastric disorders, cardiovascular diseases, pathologies of the nervous systems, cancers in all organs, ischemia, etc (Martin-Nieto and Villalobo, 1997). The identification of mutated connexin genes in Charcot-Marie-Tooth syndrome, non-syndromic senorineural deafness, cataracts, heart malformations and defects in laterality, reproductive dysfunction also contributes to the idea that modulated GJIC by environmental or genetic factors can lead to a wide variety of disparate disease states (Trosko *et al.*, 1998).

Rosenkranz *et al.* (2000) showed that, "using a method that models the properties of a large population of molecules chosen to represent the 'universe of chemicals', inhibition of GJIC is strongly linked to the carcinogenic process in rodents, to cellular but not system toxicity, to biological phenomena that may involve inflammatory processes and to development effects". This independent analysis also strongly supports the basic hypothesis of this paper, namely widely dissimilar clinical disease states can share an underlying cellular mechanism. That mechanism would include the alteration of GJIC between the stem, progenitor and differentiated cells of one tissue and those of another tissue. Depending where and when the disruption of GJIC occurs, one can get very different clinical pathologies. Modulation of GJIC during early

embryogenesis or fetal development could lead to embryo or fetal lethality or birth defects. Persistent inhibition of GJIC in tissues having initiated stem cells can lead to any organ type tumor, depending where the initiated stem cell resided. Modulation of GJIC in the eye could lead to cataracts, whereas blockage of GJIC-dependent differentiation of sperm or eggs in the testis or ovary, respectively, could lead to reproductive dysfunction. Blockage of GJIC in particular regions of the brain could lead to either acute or chronic brain or mental disorders.

V. CONCLUSIONS

The Basis of the "Biological Rosetta Stone" Concept of the Role of Modulated GJIC in "Epigenetic Toxicology"

When the real Rosetta Stone was discovered, the reductionalistic information residing in each of these sets of linguistic symbols, was by themselves, relatively unimportant. However, the insight generated by the three sets, side by side, describing the same phenomenon, allowed Jean Francois Champollion to decipher one of the sets, the Egyptian hieroglyphics, until then having unknown meanings, the discovery of GJIC by Loewenstein (1966) and its structural analogue by J. P. Revel (1988), provided a new insight that the "unit of life" of the multicellular organism was not the single cell, as postulated by Schwann, but by a syncytium of cells coupled by gap junctions. When chemicals, which were not genotoxic, could induce birth defects, cancer, immune and reproductive disorders and when it was seen that GJIC was modulated in the tissues expressing these disease states, it became obvious that the shared underlying mechanism in all these diseases was the modulation of GJIC. Since there are multiple connexins, multiple regulatory mechanisms controlling the expression and function of the connexins at the transcriptional, translational or posttranslational levels, finding a common mechanisms for any of these disease states is impossible. However, all tissues must be GJ-coupled at the cell level, regardless of how the GJIC is modulated at the molecular/biochemical/biophysical levels, modulation at the cell level is what affects control of proliferation, differentiation, apoptosis or adaptive responses of differentiated cells; in other words homeostasis must be maintained for normal development and adaptive function. Homeostasis is dependent on GJIC. Blockage of GJIC by any means disrupts homeostasis. Disrupted homeostasis can lead to diseases. Rosenkranz *et al.* (2000) make this point when they stated: "It was also surprising that irrespective of inhibition of GJIC, the observed prevalence of molecules that have the potential for jointly inducing allergic contact dermatitis, ocular irritation, sensory irritation, and respiratory hypersensitivity [teratogenesis and tumor promotion] is much greater than expected··· again suggesting a commonality between the phenomena...These findings suggest a commonality in mechanisms that is worthy of further study".

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