Connexins as targets for cancer chemoprevention and chemotherapy

Timothy J. King a, John S. Bertram b,*

a Hawaii Biotech, Inc., Aiea, HI 96701, USA
b Cancer Research Center of Hawaii, University of Hawaii at Manoa, 1236 Lauhala St., Rm. 406C, Honolulu, HI 96813, USA

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Abstract

Cells within a tissue continuously interact to coordinate normal tissue functions and maintain homeostasis. Gap junctional communication (GJC), mediated by the connexin protein family, allows this type of intercellular crosstalk resulting in synchronized and cooperative tissue behavior such as cardiac contraction. In cancer, loss of these types of cell:cell interactions has been shown to facilitate tumorigenesis and enable the autonomous cell behavior associated with transformed cells. Indeed, many human tumor lines demonstrate deficient or aberrant GJC and/or loss of connexin expression. Restoration of exogenous connexin expression/GJC function is correlated with increased cell growth control both in vitro and in vivo. In support of this growth regulatory hypothesis, decreased connexin expression has been observed in situ in early human neoplasia of various organs. Additionally, genetically engineered mice lacking particular connexins (Connexins 32 or 43) exhibit increased susceptibility to radiation and chemically-induced liver and/or lung tumorigenesis. These studies strongly suggest that connexins and GJC serve a tumor suppressor role. Consistent with this proposed role, in a model cell culture system, retinoids and carotenoids up-regulate Connexin43 (Cx43) expression in direct proportion to their ability to suppress carcinogen-induced neoplastic transformation. Here, we discuss the important role of connexins and GJC in tumorigenesis and suggest the possibility of connexins as potential anti-oncogenic targets for chemoprevention and/or chemotherapy.
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Keywords: Connexin; Gap junction; Chemoprevention; Chemotherapy; Cancer

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* Corresponding author. Tel.: +1 808 586 2957; fax: +1 808 586 2970.
E-mail address: john@crch.hawaii.edu (J.S. Bertram).

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1. Loss of GJC is common in human cancer and animal cancer models

The discovery by Loewenstein and Kanno in 1966 that cultured fibroblasts were electrically coupled when in contact was the first demonstration of the phenomenon of gap junctional communication (GJC) [1]. Loewenstein's group later showed that malignantly transformed fibroblasts were deficient in this ability to communicate and that activation of a temperature-sensitive src oncogene in communicating fibroblasts led to a rapid inhibition of junctional coupling and loss of growth control [2]. These observations led to the hypothesis of growth control through GJC [3]. In this model, GJC produces gradients of growth control signals throughout an organ, or a cell monolayer, allowing cells to control their population density. A large body of evidence now exists that implicates loss of GJC as an important event in carcinogenesis and thus identifies connexins as potential targets for prevention and possibly therapy. As discussed elsewhere in this book, gap junctions are water-filled pores formed by the docking of two hemi-channels (connexons), contributed by each of the adjoining cells (see Fig. 1). GJC allows for direct exchange between neighboring cells of small hydrophilic molecules and ions less than 1–2 kDa in size including metabolites and messengers such as sodium, potassium, calcium, cAMP/cGMP, ADP/ATP and inositol 1,4,5-triphosphate resulting in the metabolic and electric coupling of cells. Connexin function is highly regulated at the level of transcription, translation, processing, gating and protein/channel turnover. Phosphorylation of multiple serines, threonines and tyrosines, predominantly in the cytoplasmic tail, appears to contribute to the orchestration of these various regulatory steps for several...
2. Role of GJC and connexins in dysplasia and advanced cancer

Numerous studies have demonstrated that human cancers are deficient in GJC either as a result of downregulated expression of connexins, altered trafficking to the cell membrane or the inability to form functional junctions. This subject has been recently reviewed [4]. While most studies have involved human tumor cell lines, some have examined primary excised tumors, either by immunohistochemistry or by functional analysis. These studies included lung carcinomas of various pathologies, renal carcinomas, endometrial carcinomas, breast carcinomas, gliomas, cervical carcinomas and prostate carcinomas (Fig. 2). While there have been reports that melanoma tumor types continue to express connexins, this is accompanied by an altered ability for heterotypic communication; the normal communication with keratinocytes is blocked and is substituted by communication with fibroblasts [5]. This consistent lack of normal GJC in tumor cells strongly suggests that in order to progress, developing tumors must isolate themselves from the direct influence of surrounding normal cells. In this respect, connexins behave similarly to the majority of tumor suppressor genes whose expression or function must also be abrogated to allow carcinogenic progression (reviewed in [6]).

Changes in GJC during carcinogenesis have been extensively examined in skin. Human and rodent skin express multiple connexin types (Fig. 1, bottom panel). We will describe here that an additional function of this coupling is to control cell proliferation which when perturbed can accelerate the process of carcinogenesis and when restored can suppress indices of malignancy.

3. Restoration of GJC decreases the transformed phenotype

Using gene transfer technology, numerous studies have restored connexin expression to immortalized and transformed cell lines lacking connexin protein expression and observed varying degrees of cellular growth control. Responses ranged from a complete lack of effect to loss of tumorigenicity both in culture and in vivo. The variation in connexin-related effects may be due to intrinsic cell properties (mutations, etc.), clonal variation and heterogeneity (control vector-clone compared to exogenous expressing-clone), cell type-connexin type specificity or even the assay type and its relevance towards the particular growth characteristic influenced by connexin expression/GJC function.

Studies demonstrating alterations in cell logarithmic growth or saturation density suggest a role for several Cxs in regulation of cell growth parameters. One such study evaluated alterations in cell cycle regulatory proteins following re-expression of Cx43 in a canine kidney epithelial cell line [14]. In this study, altered protein levels of certain cyclins and CDKs were observed suggesting that Cx43 expression influenced cell cycle status either directly or indirectly. However, to date few definitive mechanisms for GJC/connexin-mediated cell cycle regulation or influence on tumor promotion or progression have been elucidated. One exception is the recent implication of Cx43 in the regulation of the E3-ligase Skp2 that directly mediates degradation of the cell cycle regulatory protein p27Kip1 [15,16].
The decreased Skp2 protein levels in this Cx43 expressing cell line correlated with increased p27Kip1 levels. This is in agreement with a previous study that observed increased p27Kip1 levels and enhanced growth control in a lung carcinoma and a liver carcinoma cell line engineered to re-express Cx43 and Cx32 respectively [17]. Increased p27Kip1 levels have been shown to regulate CDK/cyclin activity resulting in cellular growth arrest in late G1 phase. In humans decreased p27Kip1 expression is used as a clinical prognostic for decreased survival in a multitude of tumor types and in a genetically deficient mouse model results in increased tumorigenesis [18,19].

Increased cellular proliferation and/or loss of cell cycle regulation correlate with increased frequency of mutation fixation within the genome. As cells accrue DNA damage, control of cell cycle progression is critical to enable cellular DNA repair mechanisms to correct mutations. Loss of connexin expression/function may allow a bypass of normal cell arrest pathways resulting in diminished DNA damage repair and increased mutational frequency. In fact, one such study linked increased mutation frequency in Cx43-negative cells compared to Cx43-positive cell lines [20]. This suggests that the decreased connexin expression observed in early human dysplasia would accelerate the process of tumor progression.

Although connexin expression is lost in a majority of tumor cells, to our knowledge in only one case has a mutation within a connexin gene been reported [21]. Several studies have shown that alterations in connexin gene promoter methylation are associated with gene silencing [22]. One clinical study found promoter methylation strongly negatively correlated with Cx43 expression and decreased with distance from the primary tumor [23]. We have shown re-expression of Cx43 following treatment of HeLa cells with 5-aza-cytidine to reduce DNA methylation [24]. Additionally, observations on tumor cell lines suggest that histone deacetylation also results in connexin gene repression [22,25]. The concept of employing DNA demethylating agents or modifiers of histone acetylation status to counteract connexin gene repression is an interesting chemotherapeutic possibility which is discussed below.

Several studies have implicated viral and cellular oncogenes in altered GJC function [22]. Specifically, viral infection or transfection of viral encoded oncogenes, such as v-src and polyomavirus middle T-antigen into GJC-competent cells resulted in decreased GJC. In the case of v-src, oncogene-mediated tyrosine phosphorylation on the carboxy tail of Cx43 has been shown to decrease GJC channel function [26]. Several studies have demonstrated a link between Cx43 expression and a well studied oncogenic pathway, the Wnt signaling pathway. Investigators utilizing the intestinal tumor mouse model (min, containing a mutated adenomatous polyposis coli gene, APC) observed decreased Cx32-mediated GJC in Paneth cells of the intestine and in contrast increased Cx32 levels in stromal components of intestinal adenomas [27]. Additional studies showed that Cx43 gene expression can be upregulated via activation of the Wnt pathway and that the Cx43 promoter is responsive to β-catenin transcriptional activation [28,29]. Here, one possibility is that the APC protein plays a role in regulation of Cx32 expression in the intestine, possibly by controlling β-catenin levels/activity. It is also possible that APC deficiency allows increased cell proliferation and increased mutation frequency resulting in loss of Cx32 expression/GJC function.

4. Genetically engineered connexin mouse strains

Genetically engineered knockout mouse strains (KO mice) lacking specific connexins have recently yielded information regarding the importance of specific connexins in development as well as normal physiology for several tissue types (see [30,31] for review). For example, Cx32-KO mice display compromised liver function as revealed by defective propagation of sympathetic nerve stimulation [32], altered initiation and termination of DNA synthesis during post-hepatectomy regeneration [33] and defective signaling due to decreased propagation of inositol tri-phosphate via GJC channels [34]. These mice also exhibit altered pancreatic function, increased neural progenitor pools and peripheral nerve degeneration in older aged animals reminiscent of human CMTX [35]. Female Cx37-KO mice fail to ovulate and are therefore infertile apparently because of impaired junctional interactions between oocytes and surrounding granulosa cells leading to reciprocal impaired maturation [36]. Cx26-KO and Cx45-KO mice die in utero and Cx43-KO mice die peri-natally due to heart malformation [30,31]. This Cx43-KO effect has been shown to be the result of altered migration of neural crest cells during embryogenesis [37]. Interestingly, heterozygous Cx43-KO mice and transgenic mice over-expressing Cx43 also exhibit cardiac abnormalities further supporting the importance of regulated connexin expression [38,39]. Cx40-KO mice exhibit heart conduction abnormalities implicating a second connexin type in cardiac development and function [30]. These disparate phenotypes not only show the diversity of the expression pattern of connexins, but also illustrate the varying functions conducted by connexins and GJC in different tissues.

Mouse “knock in” experiments, in which one connexin isotype is specifically replaced with another, have shown that particular connexins play distinct roles in specific cellular processes. In a study where Cx43 was replaced by Cx32 or Cx40, the resulting mice survived and exhibited altered cardiac defects specific to Cx32 replacement, however both mouse strains also demonstrated a variety of altered developmental effects in several other tissues [40]. Recently, replacement of wild-type Cx43 with a truncated version (K258Stop) lacking the carboxy-terminal, cytoplasmic tail region resulted in perinatal lethality due to an epidermal barrier defect but not due to any heart defect as seen with Cx43-KO mice [41]. This epidermal integrity failure may be due to altered Cx43 protein trafficking, processing or degradation as the C-terminal region has been shown to contain multiple sites for phosphorylation and protein–protein interaction.

5. Cx32-KO and Cx43 heterozygous mice are more sensitive to carcinogens

Although many of the connexin KO mouse strains created have demonstrated altered development and physiology, only
Cx43-heterozygous and Cx32-KO strains have demonstrated increased susceptibility to tumorigenesis. However, it is worth noting that several KO strains (such as Cx26, Cx43 (nullizygous) and Cx45) exhibit embryonic or neo-natal lethality preventing the proper evaluation of connexin-related effects on long-term carcinogenesis [30]. Presumably, evaluation of existing or soon-to-be constructed mouse strains, in which tissue-specific ablation of these critical connexins can be achieved, would allow survival and enable investigation of cancer susceptibility.

Cx32-KO mice exhibit increased liver tumor incidence, multiplicity, size and progression (carcinoma) following exposure to chemical carcinogens (DEN, diethylnitrosamine) and radiation (X-ray) implicating Cx32 specifically in hepatic tumor promotion/tumorigenesis [42–44]. Indeed, this increased susceptibility may be related to the increased hepatocyte proliferation previously reported for this mouse model [42–44]. Mechanistically, a higher percentage of liver tumors from chemical and radiation-induced Cx32-KO mice exhibited MAPK-pathway activation compared to wild-type controls [44]. This observation suggests differential generation of mutations or selection of tumors occurs in the absence of Cx32-mediated GJC.

One carcinogenesis study showed that Cx32 also acts as a tumor suppressor in mouse lung (Fig. 3) [45]. Exposure to radiation or a chemical carcinogen (DEN) resulted in an increased tumor incidence, multiplicity and progression. This enhanced sensitivity may be due in part to the enhanced alveolar cell proliferation observed in non-transformed Cx32-KO lung tissue, similar to that observed in Cx32-KO liver [45]. Interestingly, an increased percentage of lung tumors from Cx32-KO mice exhibited MAPK pathway activation similar to that observed in Cx32-KO liver. Increased MAPK activation in both tissues suggests a modified or preferentially selected oncogenic pathway altered by the loss of Cx32-mediated GJC that is possibly held in common importance in both liver and lung tissue. Assuming a similar MAPK bias in GJC-deficient human neoplasia, the remote possibility of targeting GJC-deficient, MAPK-activated tumors using MAPK inhibitors is intriguing.

In another study, Cx32-KO mice exposed to benzene exhibited increased pulmonary pneumotoxicity but no increase in lung carcinogenesis was observed [46]. However, strain background, different carcinogen type and, as the authors admit, a small number of animals in the benzene-exposed tumor group may have contributed to discrepancies between these studies. In addition to these Cx32-related lung effects, one study observed increased lung tumor multiplicity and tumor progression (based on categorization into solid or papillary morphology) in KO mice heterozygous for Cx43 which is also expressed in the lung, [47]. The separate or combined contributions of Cx32 and Cx43, or possibly other connexins that are expressed in lung (e.g. Cx37/Cx40), to mouse lung tumorigenesis remain to be established. Further investigations utilizing double KO mice lacking both Cx32/Cx43 or conditional, lung-specific Cx43-KO mice will hopefully shed light on the exact contributions of both connexins to normal lung physiology and lung tumor character.

In both cases of KO mouse-associated increased susceptibility to tumorigenesis, it is worth noting that induction by exposure to a tumor initiator was required. Cx32-KO and Cx43-heterozygous mice do not demonstrate any increases in spontaneous liver or lung tumorigenesis [43–45]. An initial report of increased spontaneous liver tumors in the Cx32-KO
mice [42] has not been confirmed in subsequent reports by this group [43]. The lack of increased spontaneous tumor formation strongly implicates GJC in tumor promotion and/or progression in these tissues and not tumor initiation. Again, these observations in situ correlate with the observed role of GJC in culture experiments where loss of GJC-mediated interactions with normal, growth-controlled counterparts allowed autonomous growth and increased neoplastic potential [22]. Such a tumor promotional aspect to GJC-mediated tumor suppression denotes connexin upregulation as a potential chemoprevention target. The inhibitory role of GJC in liver tumor promotion is further emphasized by the observation that Cx32-KO mice are no longer responsive to the promoting effects of Phenobarbital, previously shown to inhibit Cx32-mediated GJC in this organ. In DEN-treated Cx32-KO mice, Phenobarbital failed to enhance production of both preneoplastic foci and hepatomas indicating that GJC impacts events early in the carcinogenic process [48].

6. Restoration of connexin expression in tumor cells reduces their neoplastic potential

Over the last several decades, numerous laboratories have employed constitutive gene expression systems to restore connexin/GJC function in GJC-deficient tumor cell lines resulting in variable re-establishment of several aspects of cell growth control including; decreased logarithmic growth and saturation density, decreased growth in heterologous culture with growth-controlled cells, decreased anchorage-independent growth as well as diminished in vivo tumor formation for a variety of cell types [22]. To better define connexin function in the absence of clonal heterogeneity issues, several studies have utilized inducible gene expression systems. Two such human tumor cell lines (HeLa, cervical carcinoma and HT-1080, fibrosarcoma) containing inducible Cx43 gene constructs demonstrated decreased neoplastic potential following restoration of Cx43/GJC function both in vitro (decreased anchorage-independent growth) and in vivo (decreased xenograft tumor formation) (Fig. 4) [49,50]. These studies all underscore the importance of connexins and GJC in normal cell biology and their loss in increased neoplastic potential. Although more advanced aspects of the neoplastic phenotype were diminished following GJC restoration in these cell lines, many in vitro measures of growth were unaffected. This may simply be a consequence of the prior evolution of these cell lines within the in vitro environment resulting in a loss of responsiveness to GJC-mediated signaling.

Additionally, it is worth noting that while restoration of connexin expression within the context of certain transformed cell types is associated with decreased indices of neoplasia, the extent of connexin influence is clearly limited. For example, while inducible expression of Cx43 in both HeLa and HT-1080 cell lines resulted in decreased xenograft tumorigenicity, tumors were nonetheless capable of forming in the presence of Cx43 (no loss of Cx43 expression was observed with HT-1080 tumors explanted from induced mice). Several complicating factors should be considered with respect to connexin growth control. Loss of connexin expression in fully transformed cells lines, assuming connexin loss is an early oncogenic event as it appears to be, may be accompanied or followed by multiple mutations in

Fig. 4. Inducible HeLa lines demonstrate regulable Cx43 protein expression (Western immunodetection, panel A) and increased GJC seen as increased fluorescent dye spread from microinjected cells to neighboring cells in an induced monolayer (panel C) compared to no dye transfer from numerous microinjected cells in an uninduced monolayer (panel B). Induced cells attenuated tumorigenicity in xenograft mouse experiments (nude mice) for two individual inducible HeLa clones (panels D/Clone 1 and E/Clone 2). Red dots represent mean tumor volume of induced tumors and black dots represent mean tumor volume of uninduced tumors. Numbers at end of curve represent number of detected tumors/total number of injections. P values represent statistical differences between uninduced and induced mean tumor volumes. Reproduced from King et al. [49] with permission.
other growth regulatory genes. This may create a situation where restoration of exogenous connexin expression results in only partial or no alteration of tumor growth capacity. An alternate view is that a particular connexin or connexins, in general, are capable of regulating only certain aspects of tumor progression (such as cellular density) and therefore restoration effects are limited to these specific characteristics.

Several studies have documented evidence that connexin function is tissue-type specific. As connexin type expression patterns are variable, it is possible that a particular connexin may function as a tumor modulating protein in one or several specific cell types and not in others. This may be due to expression restrictions or intracellular regulatory mechanisms (absence or presence of distinct signaling or responsive pathways). Clearly, interaction with the external environment as well as surrounding cell types increases the complexity of evaluating specific connexin function.

In addition to tumor promotion and oncogenic pathway activation, another important facet of tumor progression is vascularization and intravasation resulting in tumor expansion and metastasis. Recently, one group observed an increase in the vascularization and intravasation resulting in tumor expansion activation, another important facet of tumor progression is evaluating specific connexin function. As well as surrounding cell types increases the complexity of pathways). Clearly, interaction with the external environment may function as a tumor modulating protein in one or several patterns are variable, it is possible that a particular connexin can function as a consequence of downregulated expression of Cx43 in the engineered human cell lines described earlier [49,50].

Confusingly, several studies have presented conflicting data suggesting that connexin loss or sustained connexin expression may contribute to certain invasive properties of transformed cell lines. For some tumor types, particularly melanoma, the concept that the malignant potential is inversely correlated with GJC capacity appears to be an oversimplification. Studies of human melanoma have shown that while the capacity to communicate with keratinocytes is lost with malignant transformation, communication with fibroblasts is gained. This has been associated with a shift in cadherin expression profiles from E-cadherin, also expressed in keratinocytes, to N-cadherin expressed in fibroblasts. This shift in junctionally coupled partners was not accompanied by changes in overall expression of Cx43 in the melanoma cells [52]. In mouse melanoma cells, Cx26-mediated GJC with endothelial cells was associated with a high incidence of metastasis, while ectopic expression of Cx26 in a weakly metastatic cell line enhanced its ability to communicate with endothelial cells and increased its metastatic capacity [53]. It may be that changes in junctional partners may liberate melanoma cells from homeostatic controls, in this case mediated by keratinocytes, while at the same time allowing communication with endothelial cells and fibroblasts which may donate nutrients resulting in enhanced metastasis. Similar increases in metastatic or invasive ability have been documented for glioma [54] and HeLa cells [55] transfected with Cx43. It may be that these cell lines have lost the capacity to respond to junctionally transferred signals but may still benefit from nutrient transfer from communicating, non-transformed cells. In any event, there is a preponderance of data implicating altered connexin/GJC function in carcinogenesis in situ. The possibility of induction of connexin/GJC in combination with certain angiogenesis inhibitors or metalloproteinase inhibitors may enhance chemotherapeutic value in the clinic.

7. Cancer prevention and the role of connexins

Prevention, it is said that prevention is better than cure and this is never more true than in the case of cancer whose successful treatment in many instances has proved elusive. Opportunities for prevention occur at three distinct phases in the process of carcinogenesis; these have been termed primary, secondary and tertiary prevention (reviewed in [56]). Primary prevention concerns limiting exposure to the carcinogenic stimulus, be it tobacco, ionizing radiation or industrial chemicals. If the carcinogenic stimulus is known, this can be an effective strategy, as can be demonstrated by the current decrease in lung cancer rates following reduction in tobacco consumption. This aspect of cancer prevention will not be discussed further here. Chemoprevention, the use of chemical agents to prevent cancer development, can be applied to the secondary and tertiary forms of prevention. Secondary prevention applies to the use of strategies to prevent the formation of DNA mutations following exposure to chemical carcinogens. This has been demonstrated experimentally by the induction of enzymatic detoxification mechanisms which serve to diminish the formation of DNA-reactive metabolites. Tertiary prevention encompasses strategies which seek to prevent the further progression of cells (so-called initiated cells) possessing initial mutations which predispose them to developing subsequent mutations leading to cellular transformation. It is in this phase of carcinogenesis that connexins appear to play a role in prevention; a phase in which we have shown cancer preventive agents to cause upregulated expression of Cx43.

As discussed earlier, most human tumor cells are deficient in GJC, primarily as a consequence of downregulated expression of connexin genes. We and others have shown that this event occurs early during the carcinogenic process, i.e., during the progression of initiated cells to fully established malignant cells. In the uterine cervix, we have shown Cx43 expression to be strongly downregulated in dysplastic epithelium, presumably as a consequence of infection with HPV, the etiologic agent of most cervical carcinomas. A similar phenomenon has been demonstrated in oral leukoplakia, a preneoplastic lesion formed as a consequence of exposure to tobacco products. Similarly in the liver, in which Cx32 is predominantly expressed, progressive downregulation of expression correlates with progressive development of the neoplastic phenotype [57,58]. Further underlying the crucial role of GJC in limiting aberrant proliferation are the results obtained in the Cx32-KO mouse studies discussed above, which demonstrate greater susceptibility to carcinogen-induced neoplasia. Susceptible tissues exhibit increased rates of proliferation [42,45], an observation consistent with the widely accepted role of aberrant proliferation in the fixation of carcinogen-induced mutations associated with the subsequent progression of such
mutated cells to cancer. It may thus be hypothesized that strategies to prevent downregulated expression of connexins in initiated cells would serve to decrease their proliferation and delay their progression to neoplasia. This, while not eliminating the formation of initiated cells, would nevertheless be an effective prevention strategy if this delay could be extended to decades. The topic of connexins in cancer prevention and therapy was last reviewed in 2002 [59].

8. Retinoids and carotenoids as cancer chemopreventive agents

Retinoids are compounds which include vitamin A and synthetic vitamin A analogs. These activate nuclear retinoid receptors and serve to maintain normal growth, differentiation and immune function. Carotenoids are a group of plant-derived pigments comprised of members that can either be centrally cleaved to yield two molecules of vitamin A (pro-vitamin A, such as β-carotene) or those that cannot due to a lack of the β-ionone ring necessary for formation of retinoids (non-pro-vitamin A, such as lycopene the red pigment in tomatoes) (Fig. 5). In experimental animals and cell culture models of carcinogenesis, retinoids and both classes of carotenoid (pro-vitamin A and non-pro-vitamin A) have been shown to inhibit carcinogenesis. In clinical studies, retinoids have been shown to be effective in the prevention of cervical [60] and oral cancers [61], however, their toxicity has so far eluded successful widespread clinical application. For carotenoids, in spite of abundant epidemiologic evidence for risk reduction with increased consumption or increased blood levels of carotenoids [62,63], intervention trials have yet to convincingly show effectiveness. For example, high-dose supplementation with β-carotene in the Physicians Health Study failed to show protection against lung cancer in this relatively healthy group of individuals [64]. However, when similar studies were conducted in high-risk individuals who were currently smokers, some of whom had also been exposed to asbestos, β-carotene actually increased lung cancer rates [65]. In animal models, this toxicity was shown to be a consequence of decreased retinoid acid content and increased AP-1 signaling in the smoke-exposed lungs [66]. Conversely, in head and neck cancer, also a smoking-related disease, β-carotene has been shown to reduce the incidence of leukoplakia, a premalignant lesion [67]. It may be that the high partial pressure of oxygen in the lung predisposes this organ to the pro-oxidant action of β-carotene [68]. Additionally, the incidence of prostate cancer has been inversely correlated with dietary lycopene [62]; to test this association, two small clinical trials have been conducted in prostate cancer patients scheduled for radical prostatectomy. In both studies, there was evidence of increased apoptosis in tumor tissue [69]; moreover, one of the studies documented an increase in Cx43 expression [70]. The following discussion

Fig. 5. Chemical structures of retinoic acid (A), pro-vitamin A-carotenoid β-carotene (B), non-pro-vitamin A-carotenoid lycopene (C), astaxanthin disodium disuccinate (CARDAX™, D) and astaxanthin diphosphate (E).
will be limited to the effects of retinoids and carotenoids in model cell culture systems.

The immortalized mouse embryonic fibroblast 10T1/2 cell line was developed as an in vitro model of chemical carcinogenesis. Cells are plated at low density and exposed to chemical or physical carcinogens after which a small proportion of cells, typically 1% of survivors, acquire the characteristics of initiated cells and undergo neoplastic transformation to form a transformed focus. This occurs approximately 4 weeks after carcinogen exposure and represents clonal expansion to form a focus in the context of a complete cell monolayer (Fig. 6) [71]. The colony size achieved by initiated cells during this process of expansion is crucial to the production of transformed foci; if the colony does not reach approximately 1000 cells transformation is progressively inhibited [72]. This inhibition we now believe is a consequence of Cx43-mediated GJC between initiated cells and surrounding non-initiated cells in the monolayer resulting in decreased initiated cell proliferation. When retinoids or carotenoids were added 7 days after the initiating event and maintained for the remaining 4 weeks of the assay, a dose-dependent decrease in transformation was observed which was reversible upon drug removal [73,74]. This reversibility demonstrates that these agents do not selectively eliminate initiated cells but actually prevent their progression to neoplasia.

9. Retinoids and carotenoids up-regulate Cx43 expression and GJC in 10T1/2 cells

This chemopreventive ability of retinoids and carotenoids not only directly correlates with their ability to increase GJC among both normal and initiated cell types, but also corresponds with their capacity to upregulate GJC between the these two cell types [75,76]. We proposed a model where in the control situation, initiated cells, situated centrally in a colony of 1000 initiated cells, would be shielded from the influence of surrounding normal cells. Conversely, cultures treated with retinoids or carotenoids, with the enhanced GJC caused by these agents, would increase transfer of signal molecules from normal to centrally located initiated cells thereby preventing aberrant proliferation and neoplastic progression. Upon removal of the chemopreventive agent, GJC was shown to decrease to untreated levels thereby again effectively shielding centrally located initiated cells from the influence of surrounding normal cells. It is of interest that many classes of tumor promoters, including Phenobarbital and TPA, inhibit GJC [77]. That this activity of promoters to inhibit GJC is directly related to their tumor promoting abilities is strongly supported by the data referenced above, that Cx32-KO mice are no longer responsive to Phenobarbital in mouse liver tumor promotion experiments using the chemical carcinogen DEN [78,79]. In the 10T1/2 mouse fibroblast cell line, TPA inhibits Cx43 mediated GJC whereas treatment with retinoids counters both this inhibition and its tumor promoting action [76]. These studies strongly support the concept that an important action of tumor promoters is to downregulate GJC.

10. Carotenoids induce Cx43 irrespective of pro-vitamin A or antioxidant properties

At the time we were characterizing the cancer chemopreventive properties of carotenoids, the only known common property shared by these carotenoids was the ability to act as lipid-phase anti-oxidants, a plausible mechanism for prevention. However, when we examined the antioxidant activities of diverse carotenoids in 10T1/2 cell cultures, we discovered that although all carotenoids did indeed prevent oxidative damage, as indicated by a decrease in the formation of thiobarbituric acid reactive-substances (TBARS), this ability did not correlate with their activities as inhibitors of transformation. Furthermore, the non-carotenoid lipid-phase antioxidant α-tocopherol, was found to be a very poor inhibitor of neoplastic transformation. Even at concentrations of 10^{-4} M, 10-fold higher than that used for carotenoids, it failed to induce Cx43 expression even though it was the most effective inhibitor of TBAR formation [80]. Thus, within the limits of this particular lipid-phase oxidation damage assay, it appeared that while all carotenoids were indeed antioxidants, this did not appear to be the major factor responsible for the observed function as a transformation inhibitor. Additionally, this apparent tumor suppressive activity did not correlate with the pro-vitamin A potential of these particular carotenoids [80] as might have been expected from the similar influence of retinoids and carotenoids on Cx43 expression. Indeed, recent studies have shown that these two classes of chemopreventive agents induce Cx43 expression by different mechanisms. Gene induction at the level of protein and mRNA by retinoids but not carotenoids is inhibited by...
pharmaceutical antagonists of the nuclear retinoic acid receptors (RARs). In contrast, induction by non-pro-vitamin A carotenoids is inhibited by antagonists of the peroxisome proliferator activated nuclear receptors (PPAR) [81].

11. Problems confronting use of carotenoids as chemopreventive agents

Carotenoids are highly lipophilic molecules and are poorly and variably adsorbed after human ingestion. Additionally some carotenoids, particularly β-carotene, may become pro-oxidants under conditions of high oxygen tension such as found in the lung. Under these conditions, as first described by Burton and Ingold [68], the initial reaction with oxidant produces an unstable intermediate which can itself react with unsaturated lipids for example, to produce a chain reaction resulting in the formation of additional reactive species such as epoxides and carbonyl compounds. The biological significance of this phenomenon is unclear, but may be in part responsible for the increased lung cancer rates observed in intervention trials conducted with high-dose β-carotene in smokers and asbestos-exposed individuals [65,82]. Cell constituents are confronted with multiple sources of highly-reactive oxidative species capable of causing lipid peroxidation and DNA damage. Fortunately, not all carotenoids become pro-oxidants under these conditions, astaxanthin (ASTX, 3,3'-dihydro-4,4'-diketo-β-carotene), has been described as a more potent antioxidant than β-carotene yet does not demonstrate pro-oxidant activity under high oxygen partial pressure [83]. In order to overcome the clinical problems associated with carotenoid drug delivery and possible pro-oxidant toxicity, Hawaii Biotech Inc. (HBI, Aiea, HI) has developed novel carotenoid derivatives: including a disodium disuccinate derivative of ASTX (CARDAX™) [84,85] and a diphosphate ASTX derivative [86] (Fig. 5). Both compounds form pseudo-solutions in water at concentrations of up to 8 mg/ml (CARDAX™) and 25 mg/ml (diphosphate ASTX). Dispersibility is achieved in aqueous solution secondary to self-assembly of disodium disuccinate ASTX monomers into supra-molecular complexes. Monomeric solutions of compound can also be achieved by the inclusion of ethanol (EtOH) at concentrations up to 50%, thereby disrupting this self-assembly while preserving aqueous solubility [87].

11.1. Water-dispersible derivatives of ASTX upregulate Cx43 expression in 10T1/2 cells and potently inhibit neoplastic transformation

Development of these water-soluble compounds at Hawaii Biotech, Inc. has overcome the problems associated with delivery of dietary carotenoids to biological systems which required either tetrahydrofuran as solvent or use of a beadlet formulation (currently available for only three specific carotenoids). To determine if the disuccinate ASTX derivative CARDAX™ had potential activity as a chemopreventive, we treated 10T1/2 cells with various formulations of CARDAX™. To enhance both solubility and bioavailability, several EtOH/water formulations were tested for aggregation with UV/vis spectroscopy. The “solubility” of the derivatives was significantly enhanced by the use of 1:1 (50% EtOH) and 1:2 (33% EtOH) EtOH/water formulations. These formulations have been previously demonstrated to maintain the carotenoid derivatives in monomeric form [88]. Induction levels of Cx43, as determined by immuno-blotting, were higher with the EtOH formulation at 10⁻⁵ M than for formulations in sterile water alone, demonstrating enhanced biological availability using EtOH as a co-solvent as suggested by previous physico-chemical studies. The mixture of stereoisomers of CARDAX™ in pure aqueous formulation was able to upregulate Cx43 expression with equivalent or greater potency than that previously observed for other carotenoids in organic vehicle [75]. Importantly, treated cells were found to assemble Cx43 into immunoreactive plaques in regions of cell/cell contact, consistent with gap junction formation. This was confirmed by functional studies utilizing a dye-microinjection technique, which demonstrated that treated cells were more extensively coupled than solvent-alone control treated cells [84]. We have recently reported that the diphosphate ASTX derivative also upregulates Cx43 expression and is capable of completely suppressing carcinogen-induced 10T1/2 cell transformation at a concentration of 10⁻⁶ M, making it the most potent carotenoid we have tested in this assay [86]. The chemopreventive abilities of these compounds have not yet been explored in animal studies.

12. Connexins and chemotherapy

12.1. Exploitation of GJC for therapy: the “bystander effect”

In this context, the “bystander effect” refers to the ability of mutant or transfected cells to modulate the behavior of surrounding cells (bystanders). First noticed in cell cultures used to study induced mutations conveying resistance to ouabain or to 5-aza-guanine, it was discovered that in the former instance, wild-type cells surrounding mutant cells were capable of rescuing the mutants from toxicity [89]. This phenomenon is now known to be due to the reciprocal transfer of sodium and potassium ions, and occurs only when these neighboring cells are in junctional communication. Because ouabain poisons the sodium/potassium ATP pump and leads to cell death, it was apparent that the mutant cells were capable of taking over the functions of the poisoned enzyme in the wild-type cells (termed the “kiss of life”). In the second example, the inverse phenomenon appeared; wild-type cells poisoned the communicating adjacent mutant cells which would normally not be susceptible to the cytotoxic drug. This was called the “kiss of death” where cytotoxicity resulted from the transfer of phosphorylated 5-aza-guanine from Wild-type cells through gap junctions to mutant cells incapable of phosphorylating 5-aza-guanine to its active cytotoxic metabolite. Since the phosphorylated nucleotide is charged, it cannot normally pass through the plasma membrane and can only gain access to mutant cells if those cells are in direct junctional communication. This ability of communicating cells to transfer lethal
metabolites has been exploited in experimental cancer chemotherapy, unfortunately, clinical trials using this methodology have yielded limited success [90].

While this type of experimental therapy has been most extensively employed to treat gliomas, recently, attention has also been given to treatment of ovarian carcinoma and melanoma, all tumors notoriously difficult to treat by conventional chemotherapy or radiotherapy. Here, gene therapy relies on the ability of viral vectors to deliver the herpes simplex version of the gene thymidine kinase (HSV-\(tk\)) to implanted tumors followed by treatment of patients with the anti-viral drug ganciclovir (GCV) (Fig. 7). Mammalian cells cannot phosphorylate GCV to a cytotoxic nucleotide; however, herpes-infected cells, or in this case cells transfected with HSV-\(tk\), are able to phosphorylate the drug to an active cytotoxic metabolite. The “bystander effect” was evident by the observation that the number of cells killed was several fold higher than the number of cells successfully transfected with the herpes transgene. Subsequently, it was discovered that this phenomenon was directly correlated with the extent of GJC between transfected tumor cells and surrounding non-transfected cells and is attributed, as in the earlier observations with mammalian HGPRT mutants, to the transfer of cytotoxic, charged nucleotides (GCV) through gap junctions [91]. Clinical failure of this novel therapy is probably a result of the notoriously inefficient delivery of viral vectors to solid tumors. In an attempt to circumvent problems of gene delivery, the use of genetically engineered cells has been explored. As an example, neural stem cells, which are known to be capable of extensive migration through the brain, were stably transfected with HSV-\(tk\) and mixed with primary or established cultures of human glioma cells. Approximately one transfected cell was shown to be capable of killing ten tumor cells; an effect dependent upon cell/cell contact and proportional to the extent of GJC between transfected tumor cells and surrounding non-transfected cells. This combined effect resulted in a dramatic increase in the “bystander effect”, i.e., more efficient killing of HSV-\(tk\) negative glioma cells [93]. As discussed previously, most tumor cells appear to down-regulate Cx43 expression as a result of DNA methylation and gene silencing. Since histone deacetylation is strongly linked to DNA methylation, it would appear that expression of both genes (endogenous Cx43 and the transfected viral gene) was most likely being inhibited by epigenetic mechanisms.

A variation of this approach has recently been explored as a potential treatment for ovarian cancer. This cancer is known to extensively metastasize to the abdominal cavity resulting in very poor prognosis. These investigators, utilized mesothelial cells, which are easily harvested from human peritoneal cavities, are known to be capable of forming viable cell colonies on re-injection into the peritoneal cavity and, importantly, are capable of directly interacting with ovarian carcinoma cells. Human mesothelial cells, genetically engineered to express HSV-\(tk\), were shown to be capable of killing human ovarian carcinoma cells in culture and in a xenograft mouse model resulting in increased life span in xenografted mice when exposed to GCV [94]. This offers the interesting prospect of utilizing isologous mesothelial cells to deliver a cytotoxic metabolite to peritoneal metastases, thus avoiding use of immunogenic cells or plasmids. However, distant metastases would not be affected.

13. GJC as a therapeutic target

In earlier sections of this chapter, we have described that GJC is extensively downregulated in the vast majority of human tumors. Moreover, in the experiments in which GJC has been reestablished by expression of exogenous Cx43 genes, the neoplastic phenotype of these human and animal cells has been strongly inhibited. In cell culture, this is most apparent as a dramatic decrease in anchorage-independent growth; in vivo this
is observed as decreased growth of transplanted tumors. As with other tumor suppressor genes, downregulated expression of connexins appears most frequently as a consequence of gene silencing through DNA methylation. As we [24] and several other investigators have shown, use of histone deacetylase inhibitors, or of 5-aza-cytidine, an inhibitor of DNA methylation, either singly or in combination, causes re-expression of silenced genes including connexins. If this could be achieved clinically, this might cause the dramatic decrease in tumor growth rates observed experimentally after forced expression of connexin genes.

A second approach would be to use the growth suppressive effects of GJC to inhibit the growth of micrometastases. This has been shown to be effective in cell culture and in one experimental animal model of lung metastasis. This approach also requires the presence of a pharmacological agent to restore GJC between tumor cells and normal cells. Here, drug treatment employs agents that inhibit cyclic AMP phosphodiesterase (cAMP-PDE) thereby elevating intracellular levels of cAMP. This phenomenon was first demonstrated in co-cultures of non-transformed mouse 10T1/2 cells and malignantly transformed derivatives of this cell line. In the control situation, when transformed cells are allowed to attach to the growth-inhibited monolayer of normal cells, the transformed cells are capable of colony formation and progressively invade the normal monolayer. However, if cultures are treated simultaneously with cAMP-PDE inhibitors, the malignant cells attach but fail to proliferate for as long as drug treatment is maintained. Upon withdrawal, cells rapidly reenter the cell cycle and proceed to invade the normal cells [95]. The extent of growth inhibition was shown to be directly proportional to the degree with which the two cell types were in junctional communication [96]. 10T1/2 cells are known to express Cx43 whose C-terminal tail contains multiple serine phosphorylation sites known to be essential for functional communication, and it must be assumed, but this has not been demonstrated, that elevated cAMP levels increase the phosphorylation state of crucial serine residues.

Inhibition of lung metastasis was observed in an experimental mouse model involving the highly metastatic Lewis lung carcinoma cell line. In the control situation, after either intravenous injection of these cells or after spontaneous shedding from a transplanted intramuscular tumor, carcinoma cells rapidly form discrete lung metastases which can be readily quantified. However, when animals were treated with the same cAMP-PDE inhibitors shown to inhibit growth of both transformed 10T1/2 cells and Lewis lung carcinoma cells when in co-culture with growth inhibited normal cells, lung metastases were virtually abolished for the duration of drug treatment. Following drug removal, as in the cell culture studies, tumor cells rapidly grew to form macroscopic metastases [97]. Pathological analysis of lungs of treated animals revealed the presence of micrometastases which were clearly unable to proliferate. By using pulse-chase radiolabeled thymidine, we were able to show that these carcinoma cells were growth arrested with a prolonged G1/G0 cell cycle phase [98]. While this approach does not eliminate the tumor cells, it prevents their growth to form life-threatening metastases as shown by the dramatic increase in life span of treated animals. If this were translated clinically, it could be used to prevent the proliferation of tumor cells released from the primary tumor at time of surgery, for example, until a time when the patient may be better able to withstand conventional chemotherapy. At this time treatment with the cAMP-PDE inhibitor would be withdrawn, tumor cells would rapidly enter the cell cycle and then become susceptible to currently available cycle-specific therapeutic agents.

14. Concluding remarks

The consistent demonstration of loss of functional GJC as a consequence of abnormal expression or aberrant processing of connexins, together with data showing that restoration of GJC reduces the neoplastic potential of human and animal cancer cells, clearly establishes connexins as tumor suppressor genes. Importantly, increased tumor susceptibility in mice deficient for particular connexins further supports the proposed tumor suppressor role of connexins and GJC. In pre-malignant cells, reduced expression may be normalized by chemopreventive agents such as the retinoids or carotenoids. Here, restoration of GJC may also restore growth control. However, in fully malignant cells containing multiple mutations, restoration of GJC appears to require either forced expression of connexins by gene transfer or use of drugs to hypo-methylate silenced endogenous connexin genes. Frequently, in cell culture, malignant cells appear to loose only the most advanced aspects of the malignant phenotype; anchorage-independent growth and growth as xenografts. The mechanism by which connexins modify cell behavior is incompletely understood and may lead to the identification of novel pathways and novel targets for prevention and therapy. The exploitation of GJC to allow the selective transfer of toxic metabolites to target cancer cells, described as the “bystander effect”, is a promising development in cancer therapy that will also benefit from an increased mechanistic understanding of connexin gene expression regulation.

Acknowledgements


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