Role of Gap-Junctional Communication in Breast Cancer Progression and Chemoprevention\textsuperscript{1,2}

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Although the etiology of breast cancer is varied, it is likely that the modulation of oncogene and tumor suppressor gene expression may play an important role in mammary tumorigenesis. Consistent with this possibility is the finding of gene amplification or the enhanced expression of various oncogenes in primary human breast tumors and breast tumor cell lines (Callahan 1987, Callahan and Campbell 1989). Overall, these human tissue and animal studies suggest that mammary tumors arise in a multistep process and may involve the activation and inactivation of multiple oncogenes and tumor suppressor genes.

Tumor suppressor genes are considered likely to be important in human breast cancer (Marshall 1991, Sager 1992). In contrast to oncogenes, tumor suppressor genes inhibit cell proliferation, and their loss is associated with tumorigenesis. A screen for tumor suppressor genes from human mammary epithelial cells resulted in the cloning of a gene encoding a gap junction protein (Lee et al. 1991). It has long been speculated that gap junctions mediate cell-cell interactions important in growth suppression and regulation (Loewenstein 1979). For example, coculturing of transformed cells with normal cells can suppress cell proliferation and other characteristics associated with the transformed phenotype (Mehta et al. 1986, Stoker et al. 1966). In one study, growth suppression was correlated with the efficiency of gap-junctional coupling between the transformed and nontransformed cells (Mehta et al. 1986). Other studies also showed that the transfection of connexin gene expression vectors into tumor cells can restore normal cell growth in vivo (Hirischi et al. 1996, Mehta et al. 1991, Zhu et al. 1991) and reduce tumorigenicity in vivo (Eghbali et al. 1991, Rose et al. 1993).

Gap junctions are composed of proteins known as the connexins (Bennett et al. 1991, Beyer et al. 1990, Kumar and Gilula 1992). One of the most widely expressed connexin genes, connxin (Cx) 43, is also found in the mammary epithelium (Lee et al. 1991 and 1992), a connexin gene often coexpressed with Cx43 or Cx32. Moreover, gap junction gene expression in human breast cancer tissue and cells is consistently down-regulated (Lee et al. 1992, Wilgenbus et al. 1992). A role for gap-junctional perturbation in mammary tumorigenesis is suggested by the finding that all mammary tumors are deficient in gap junctions (Wilgenbus et al. 1992), and cells derived from mammary tumors do not exhibit gap junction–mediated cell-cell communication (Lee et al. 1992). These findings further indicate the possible importance of gap junctions in the regulation of mammary epithelial cell proliferation.

Chemoprevention is a new area of breast cancer research that shows great promise. In particular, tamoxifen, progestins, and retinoids have been shown to be effective in lowering the risk of breast cancer (Dickens and Colletta 1993). Another class of compounds that may be useful for breast cancer chemoprevention is the trypsin and chymotrypsin protease inhibitors derived from various vegetables. The efficacy of protease inhibitors in cancer prevention is well documented (Kennedy 1993a). For example, protease inhibitors have shown the capacity to suppress carcinogen-induced malignant transformation of tissue culture cells (Kennedy 1993). There is also a large body of data from animal studies that demonstrates the anticarcinogenic activity of protease inhibitors (Kennedy 1993).

In this study, we examined a soybean-derived protease inhibitor, the Bowman-Birk inhibitor (BBI), as a possible breast cancer chemopreventive agent. Bowman-Birk inhibitors suppress tumorigenesis in several experimental carcinogenesis model systems (Kennedy 1993a). In addition and of particular significance to these studies is the observation that a soybean diet rich in protease inhibitors (e.g., BBI) lowered the breast tumor incidence in irradiated rats (Troll et al. 1980). Our interest in BBI is further motivated by several considerations of a more practical nature. First, BBI, a product derived from a natural food source (soy beans), is likely to be nontoxic to humans. Second, the animal studies of Kennedy and coworkers (Kennedy 1993b, Oreffo et al. 1991) have shown the efficacy and safety of BBI as a cancer chemopreventive agent. Third, these studies also demonstrated that BBI can maintain its cancer chemopreventive potency even when administered as a dietary supplement (Kennedy et al. 1993). Hence, it is likely that BBI will be eminently suitable as a cancer chemopreventive agent for use in humans.

Although the mechanisms by which protease inhibitors suppress carcinogenesis are unknown, they are thought to act...
as antipromotional agents in many of the in vitro and in vivo two-stage carcinogenesis experimental models (Kennedy 1984, Troll et al. 1970). The suppression of malignant transformation by BBI and other anticarcinogenic protease inhibitors has been suggested to be mediated via their effects on the expression of c-myc and other genes or oncogenes involved in the initiation, promotion or progression of the malignant phenotype (St. Clair and St. Clair 1991).

In this paper, we report our finding that the expression of a gap junction gene Cx43 is induced in human mammary epithelial cells after BBI treatment. This observation is particularly intriguing because gap junctions are proposed to play a role in growth suppression and tumor promotion. Moreover, as mentioned above, human breast cancer cells have been shown to be deficient in gap junctions (Lee et al. 1992, Wilgenbus et al. 1992).

**MATERIALS AND METHODS**

**Cell culture.** Cells were grown in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and antibiotics (penicillin, 100 U/mL; streptomycin, 100 mg/mL). Each cell line was cultured under two different conditions. The control condition was growth in their respective media. The treated condition was growth in their media with the addition of BBI (100 μg/mL). Cells were grown to 80% confluence and then starved of serum for 24 h. They were subsequently serum stimulated for 4 h in medium containing twice (20%) the initial serum concentration. The cells were scraped, pelleted and flash-frozen at the end of the 4 h for subsequent RNA isolation for Northern analysis.

**Northern analysis.** We isolated total cellular RNA by the RNAzol B Method (Cinna/Biotex, Houston, TX). Total RNA (10 μg) were denatured and resolved by agarose gel electrophoresis and then blotted onto Duralose-UV nitrocellulose membranes (Stratagene, La Jolla, CA). Those blots were hybridized to human Cx43 riboprobes. In addition, each lane was loaded with 10 μg of total RNA, and stringency to permit visualization of the 18S ribosomal RNA band.

**RESULTS**

*Modulation of connexin 43 expression by Bowman-Birk inhibitor.* In preliminary experiments, we observed by Northern blot hybridization that treatment of a rodent fibroblast cell line (C3H/10T1/2) with the protease inhibitor BBI resulted in a four- to fivefold increase in the expression of transcripts from the gap junction gene Cx43 (Fig. 1A, B). A similar induction of Cx43 transcripts was seen when these cells were treated with a synthetic protease inhibitor, Antipain (Fig. 1A, B). In contrast, we noted that there was only a low level of Cx43 transcripts in the radiation-transformed C3H/10T1/2 cell line (Fig. 1B), a characteristic of transformed cell lines in general. We also determined that the BBI-mediated increase in Cx43 transcripts was correlated with an increase in the level of gap-junctional coupling in BBI-treated C3H/10T1/2 cells (data not shown). This was demonstrated with the examination of dye coupling using intracellular microelectrode impalements to inject the fluorescent dye tracer carboxyfluorescein. These microelectrode impalement studies showed that the BBI-treated cells exhibited an increased level of gap-junctional communication, as indicated by the very extensive spread of the fluorescent dye tracer. In comparison, the non-treated cells exhibited only a low level of dye transfer.

We also examined the effects of the protease inhibitor BB on Cx43 expression in the human mammary epithelial cell lines, MCF-10, MCF-7 and BT-20. MCF-10 is a spontaneously immortalized mammary epithelial cell line derived from human fibroblastic mammary tissue. It is nontumorigenic and likely represents cells that have been initiated along the path to malignancy but are still premalignant (Soule et al. 1990). In contrast, MCF-7 and BT-20 are tumorigenic cell lines derived from human breast cancer tissues (Soule et al. 1973). Northern blot analysis revealed that BBI treatment of MCF-10 led to an induction of Cx43 transcript expression, whereas no...
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**FIGURE 2** Northern blot analysis of RNA isolated from various human mammary epithelial cell lines. Each lane was loaded with 10 μg of total RNA, and hybridization was carried out using a 32P-radiolabeled human Cx43 cDNA riboprobe (transcribed from plasmid HCJ2; obtained from G. Fishman, Albert Einstein College of Medicine, N.Y.). Note that although the Cx43 transcript (3.2-kb band denoted by arrowhead) was observed only in the MCF-10 sample treated with Bowman-Birk inhibitor, the same amount of RNA was loaded in each lane. This was confirmed by the finding of a similar ethidium bromide staining intensity in each lane for the two rRNA bands.

Cx43 transcripts were detected in MCF-7 or BT-20, either with or without BBI treatment (Fig. 2). These findings are particularly intriguing because in previous studies, BBI was shown to exert its anticarcinogenic effects only at the initiation and prometastatic stages of tumor progression, with no suppressive effects elicited on fully malignant cells or tumors (Kennedy 1984, Troll et al. 1970).

**DISCUSSION**

These observations suggest that gap junction genes may indeed be a target for regulation by BBI. Moreover, the up-regulation of gap junction gene expression by BBI is consistent with the observations from Sager’s laboratory (Lee et al. 1991), suggesting that gap junctions may be categorized as tumor suppressor genes. Overall, these preliminary results suggest that the MCF-10 mammary epithelial cell line is an excellent model system for examining the effects of BBI on gap junction gene expression. We hypothesize that gap junctions may play a role in early events in the progression of mammary tumorigenesis and that these events may be modulated by protease inhibitors.

**ACKNOWLEDGMENT**

I wish to acknowledge Cecilia Lo, University of Pennsylvania, for carrying out the functional cellular communications experiments.

**LITERATURE CITED**


