BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 64–69 (1996)
ARTICLE NO. 1468

Reduced Connexin 43 Expression in High Grade, Human Prostatic Adenocarcinoma Cells

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Received July 18, 1996

Gap junction-mediated communication is required for normal cellular growth and differentiation. As cancer is thought to be a manifestation of the breakdown of cell-cell communication, with the concomitant loss of growth control, it would be expected that alterations in the primary structure, processing, oligomerization or trafficking of connexin (cxn) molecules would have a profound effect on the neoplastic process. Here we a present a preliminary immunohistochemical and molecular analysis of cxn 43 expression in prostatic epithelial cells from resected human tissue. Our data indicate that benign prostatic epithelial cells express cxn 43 protein, but that this expression is diminished in more advanced, anaplastic cancer cells. These data suggest that decreased connexin expression is not involved in the initiation of prostate cancer, but rather occurs during the progression of the disease.

Gap junctions play a central role in cell-cell communication and signal transmission by facilitating the exchange or passage of ions and small molecules between cells (1, 2). Structurally, gap junctions are intercellular aqueous channels, consisting of two membrane-spanning hemichannels (called connexons), one on each interacting cell (1, 2). Individual connexons are composed of multiple connexin molecules. Connexins are encoded by a multigene family, whose expression exhibits cell, cell cycle, stage-specific, temporal and tissue preference (1, 2).

As cancer is thought to be a manifestation of the breakdown of cell-cell communication and the loss of inherent growth control, it is not surprising that alterations in the primary structure, processing, oligomerization or trafficking of connexin molecules would have a profound effect on the neoplastic process. The aberrant or reduced expression of various connexin molecules has been observed in numerous cancer cells or cell lines such as human bladder cancer cells (cxn 26) (3), human keratinocyte cell lines (cxn 43) (4), and rat hepatocarcinoma cells (cxn 32) (5). Interestingly, the treatment of normal cells with tumor promoters such as PCB 126 decreases the expression of cxn 26 and cxn 32 in rat liver cells, probably via a post-transcriptional process (6, 7). Conversely, the restoration of cxn protein expression by molecular means has been shown to cause cancer cells to revert to a more normal phenotype (8, 9).

As the population of America ages, prostate cancer is emerging as a major health problem. Yet, little information is available on the biology of normal prostatic epithelial cells, such as an analysis of the expression of various connexin molecules in the normal, precancerous and cancerous state. Here we a present a preliminary immunohistochemical and molecular analysis of cxn 43 mRNA expression in prostatic epithelial cells from resected human tissue. Our data indicate that more advanced cancerous epithelial cells exhibit a reduced expression of cxn 43, suggesting a role for diminished expression of cxn 43 in the progression of prostate cancer.

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Abbreviation used: cxn, connexin.

0006-291X/96 $18.00

MATERIALS AND METHODS

Surgical specimens. Prostatic tissue was obtained from radical prostatectomy specimens resected at the Montefiore Medical Center, Bronx, NY. None of the patients received hormonal therapy prior to radical prostatectomy. Specimens were sectioned fresh and reviewed by the Department of Pathology for areas grossly consistent with cancerous and benign tissue. A section of tissue was taken from each of these areas and flash frozen in liquid nitrogen. In addition, a ''mirror image'' tissue section was fixed in 10% buffered formalin, embedded in paraffin and cut into 5-6 micron sections and stained with hematoxylin and eosin.

RNA preparation and Northern blot analysis. Whole RNA was prepared from flash frozen prostatic tissues using the Trizol procedure (Life Technologies, Gaithersburg, MD). Whole RNA was size fractionated on agarose-formaldehyde gels, transferred to GenScreen, and hybridized with [^{32}P]-labeled oligonucleotide probes as previously described (10). The intensity of hybridization bands on autoradiography film was quantitated using a Molecular Dynamics Densitometer. The sequence of the cxn 43 specific probe is 5′-AAGATGGTTTTCTCCGTGGG, corresponding to the complement of nucleotides 809-830 of the human cxn 43 cDNA (11). The hybridization and washing temperature was 52°C. The sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe is 5′-AGGACGTGGTGG-TTGACGAAT, corresponding to the complement of nucleotides 445-465 of the human GAPDH (12), and was used at 60°C.

Immunohistochemistry. Immunohistochemistry was performed using the streptavidin-biotin technique. Sections of 5 micron thickness were cut and placed on poly-1-lysine coated slides and dried in an oven at 59°C overnight. The slides were then deparaffinized in xylene and then rehydrated in decreasing concentrations (100%/ 95%/ 80%/ 70%) of ethanol. Endogenous peroxidase was blocked by immersion of the slides in 3% hydrogen peroxide at 37°C for 30 minutes. An anti-connexin 43 monoclonal antibody (anti-cxn 43) was obtained from (Zymed Laboratories, Inc., San Francisco, CA). This antibody was produced in a mouse against a 19 amino acid synthetic peptide. Sections were then incubated with the anti-cxn 43 antibody (100ug/100 ul) at a dilution of 1:25 with phosphate-buffered saline (PBS)(pH 7.4) for 2 hours at 4°C. The slides were rinsed with PBS and then incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes. After rinsing with PBS, an avidin-horse-radish peroxidase H complex was added to each slide for 30 minutes. The chromagen, 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) was added to each section for 5 minutes. The slides were sequentially rinsed in water and PBS, counterstained with Mayer's Hematoxylin (Sigma, St. Louis, Mo) for one minute, dehydrated, and coverslipped with Permount (Fisher, Fair Lawn, NJ). Sections of tissue containing smooth muscle were stained as a positive control (data not shown). Negative control prostatic tissue, processed as above, but

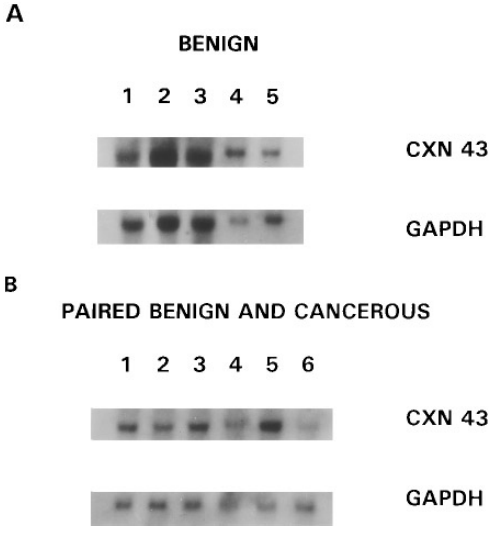
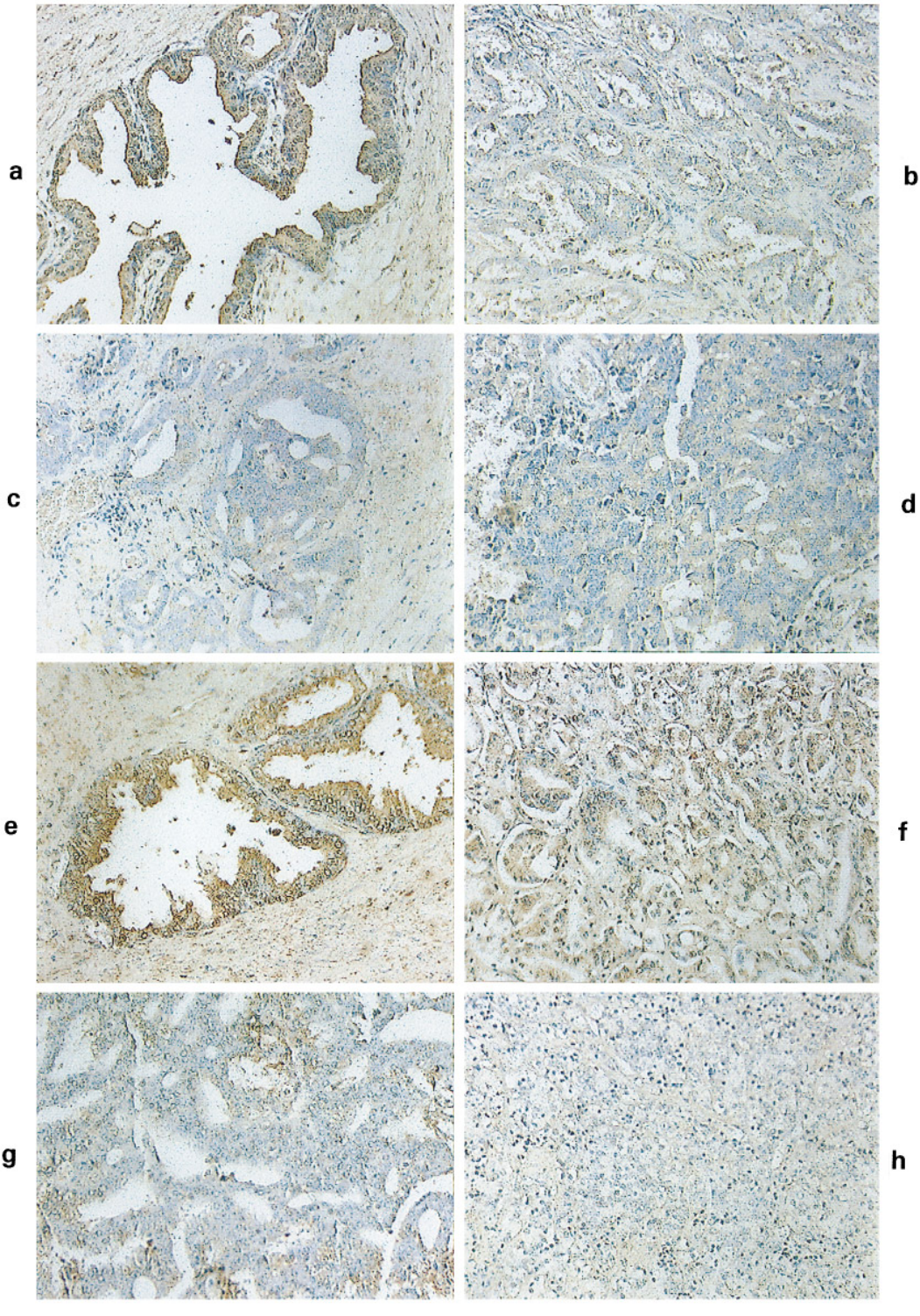


FIG. 1. Hybridization analysis of prostate RNA. Whole RNA was size fractionated on agarose-formaldehyde gels, transferred to GenScreen and hybridized with [^{32}P]-labeled oligonucleotide probes for cxn 43 and GAPDH, as previously described (10). (A) RNA from benign prostates. Each lane represents RNA from a different patient. (B) Matched pairs of RNA of benign and cancerous tissue from three patients. Lanes 1, 3 and 5; benign. Lanes 2, 4, 6; cancerous. Cxn 43 mRNA migrates at approximately 3000 nucleotides; GAPDH migrates at approximately 1500 nucleotides.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without primary antibody was included in all experiments (data not shown). Stained sections were examined on a Zeiss Axioplan microscope equipped with a video camera, monitor and printer, at the Albert Einstein College of Medicine Cancer Center Analytical Imaging Center.

RESULTS

Connexin 43 expression in the prostate was demonstrated by hybridization analysis of RNA from portions of benign prostatic tissue from 5 patients with a cxn 43 specific probe, which revealed a single band at an expected molecular size of approximately 3000 nucleotides (Fig. 1A). When normalized for GAPDH expression, there is some variation in the level of cxn 43 RNA from patient to patient (Fig. 1A). The integrated areas of intensity of cxn 43 expression (normalized to GAPDH) range from 344 to 1117 with a mean of 689.8 $\pm$ 258 (mean $\pm$ standard deviation). This variation may reflect actual quantitative differences in cxn 43 between individuals due to age or other factors, or qualitative differences, such as the representation of different prostatic cell types in the tissue samples used. Thus, in contrast to the observation that cxn 43 mRNA is not synthesized in the rat prostate (13), we have observed cxn 43 mRNA in all human prostate samples assayed.

To determine if the expression of cxn 43 RNA is decreased in prostate cancer, paired benign and cancerous tissue from three patients were obtained and analyzed for the relative expression of cxn 43 RNA. As depicted in Figure 1B the level of cxn 43 RNA is reduced in all three cancerous samples (lanes 2,4 and 6), as compared to their paired benign samples (lanes 1,3 and 5). When normalized for GAPDH mRNA intensity (Fig. 1b), cancerous samples 2, 4 and 6 exhibit an approximately 21%, 25% and 75% reduction in cxn 43 mRNA expression, respectively, as compared to benign samples. Surgical pathology reports indicate that samples 2, 4 and 6 contain prostatic cancer lesions of the Gleason grades 7 (3+4), 5 (2+3) and 6 (3+3), respectively. Although we have analyzed only a small number of samples, the data are suggestive of a decreased expression of cxn 43 mRNA in cancerous prostate tissue.

Using a cxn 43 specific monoclonal antibody, an immunohistochemical analysis was performed on formalin fixed, paraffin embedded sections of benign and cancerous tissue from three patients. Both stromal and epithelial cells stained positively for cxn 43. However, staining for cxn 43 was more intense in benign prostatic epithelial cells than in stromal cells. Despite patient to patient variability, in all three prostates analyzed, cancerous tissue stained less intensely than benign tissue. Using benign tissue in each slide as the positive standard, and stromal staining as baseline, cancerous tissue of higher Gleason grades exhibited reduced cxn 43 expression as compared to benign tissue.

Figure 2 displays immunohistochemically stained sections from two patients. Benign tissue from patient A (Fig. 2a) stains well with the anti-cxn 43 antibody, but the cancerous tissue (Fig. 2b - grade 3; Fig. 2c - grade 3, cribiform pattern; Fig. 2d - grade 4) exhibit a reduced staining intensity. Likewise, in patient B, a decrease in staining intensity was also seen in benign (Fig. 2e) vs. cancerous tissue (Fig. 2f-h). Interestingly, in this patient, the intensity of staining of grade 3 carcinoma appears slightly reduced (Fig. 2f); of grade 3 cribiform carcinoma, moderately reduced (Fig. 2g); and of grade 5 carcinoma, greatly reduced (Fig. 2h). Similar results were obtained with prostatic tissue from a third patient. Immunohistochemical analyses of smooth muscle tissue (positive control) and negative controls without the primary, anti-cxn 43 antibody gave the expected results (data not shown).

FIG. 2. Immunohistochemical analysis of prostate tissue with an anti-cxn 43 monoclonal antibody. Patient A, benign tissue (a); grade 3 lesions (b); grade 3 cribiform structures (c); grade 4 foci (d). Patient B, benign tissue (e); grade 3 foci (f); grade 3 cribiform structures (g); grade 5 epithelial cell sheets (h). All magnifications with 20$\times$ objective. Immunohistochemical analyses of smooth muscle tissue (positive control and negative controls without the primary, anti-cxn 43 antibody gave the expected results) (data not shown).

DISCUSSION

Intercellular communication via gap junctions plays a central role in tissue organization and the maintenance of the normal phenotype (1, 2). Conversely, the disruption of intercellular communication is thought to be a significant factor in neoplastic transformation (1, 2). Our data suggest that, for the limited set of paired benign and cancerous prostate tissue samples, a reduction in the steady state level of cxn 43 occurs in neoplastic prostatic tissue. While it is unknown whether the decrease in cxn 43 expression results in functional alterations in prostate cancer cells, our data are consistent with the notion that neoplastic transformation is associated with decreased intercellular communication. Further analyses are necessary to determine the full spectrum of connexin molecules expressed in normal and cancerous prostatic epithelial cells and their phosphorylation state. Still to be performed are physiological studies such as dye transfer and electrophysiological measurements to determine the functional effects of the apparent decrease in cxn 43 expression.

While cancerous tissue exhibited reduced staining for cxn 43, even anaplastic epithelial cells are not totally devoid of cxn 43 protein. While it is difficult to draw conclusions from data from only three patients, it appears that Gleason grade 3 foci are variable in cxn 43. This may reflect the heterogeneity of lesions classified as grade 3. Further, grade 3 lesions are at a transition point, between cells in acinar structures and the more disorganized grades 4 and 5. Our data indicate that the reduction in cxn 43 expression most closely parallels the loss of acinar structure, as in cribiform grade 3 and grades 4 and 5. It remains to be determined where in the neoplastic process the observed decrease in cxn 43 expression has its effect. The finding that cxn 43 expression is reduced most significantly in more advanced lesions, particularly those where acinar structure is lost argues for its role, not in the initiation of cancer, but perhaps affecting the progression of the disease, from a low grade, latent tumor to a higher grade and more aggressive cancer. The potential prognostic value of cxn expression in prostate cancer remains to be determined.

Of considerable concern is the observation that testosterone has been shown to increase the expression of connexin 32 in spinal cord motor neurons (1). As androgen ablation is the only current treatment (though temporary) for metastatic CaP, the possibility exists that the withdrawal of testosterone aggravates the disease by further decreasing intercellular communication. Further analyses into the effect of sex hormones on connexin expression in normal and cancerous prostate cells is warranted. Further, the ability of carotinoids and retinoids to increase cxn 43 expression in fibroblasts suggests that future phamacologic intervention strategies may consider the use of these and similar agents to help revert the neoplastic phenotype (1).

ACKNOWLEDGMENTS

This work was supported in part by a grant to J.G. (P30-CA-13330). The authors greatly appreciate the contribution provided by Ernie Middleton who sectioned all the paraffin-embedded tissue used in this study. The authors also thank Frank Macaluso, Leslie Gunther, and William Schubert of the AECOM Cancer Center Analytical Imaging Center for their help in using the Zeiss Axioplan microscope and video equipment.

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