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Early prostate cancer detected using expression of non-functional cytolytic $P2X_7$ receptors

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Aims: To detect early prostate cancer reliably by monitoring the expression of non-functional $P2X_7$ cytolytic purinergic receptors.
Methods and results: $P2X_7$ receptors were absent from normal prostate epithelium obtained from post mortem tissue and tissue from cases of transurethral resection collected from young men ($n = 23$) who were confirmed to be free of cancer at later procedures 5–10 years after collection of the original samples. However, $P2X_7$ was present in every case of 116 confirmed prostate cancers regardless of Gleason grade or patient age. $P2X_7$ was present in apparently normal epithelial cells in acini well outside the tumour margins, but appeared in a distinct stage-specific manner commencing with the nucleus, progressing to the cytoplasm and collecting finally on the apical membrane of the epithelial cells in morphologically distinct cancer. The pattern of $P2X_7$ receptor localization in the epithelial cells was recorded in earlier biopsies obtained from the same patient cohort. One hundred and fourteen of 116 prostates stained positively for $P2X_7$ at the earliest biopsy, though generally with a less advanced pattern of distribution.
Conclusions: The appearance of $P2X_7$ receptors in normal prostate tissue adjacent to prostate tumours makes direct tumour biopsy less critical for positive cancer diagnosis and enables cancer progression to be monitored.

Keywords: prostate cancer, diagnostic, $P2X_7$ calcium channel receptor, apoptosis, immunolabelling

Abbreviations: PIN, prostatic intraepithelial neoplasia; PSA, prostate specific antigen

Introduction

Prostate cancer is the second commonest cause of cancer death in males after lung cancer in Western society. Although the incidence of prostate cancer is increasing in many countries, there is a lack of reliable prognostic indicators able to predict the behaviour of individual tumours. Latent prostate cancer and the preinvasive form of neoplasia known as prostatic intraepithelial neoplasia (PIN) have been found in 45% of autopsy specimens following death from trauma from men in their 50s to 82% of men aged in their 80s.[1] In Australia, a total of 12 500 new cases of prostate cancer were detected in the year 2000, of which 8000 were already well advanced with medium- to high-grade Gleason score and with many (20–30%) metastatic, a similarly high rate to other Western countries.[2] The advanced stage of the disease at initial diagnosis often results from the difficulty of detecting tumours and to cases in which the onset of disease is rapid. Thus, more effective early detection of clinically significant cancers is a high priority, particularly among those men who present with elevated prostate specific antigen (PSA) and who appear negative for cancer at initial biopsy. In the same way that $P2X_7$ receptor detection has been shown to differentiate between cases of aggressive and more benign breast cancers[3] both lobular and ductal, it is hoped that a

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similar detection system will prove useful in prostate cancer. In addition, the $P2X_7$ might be valuable in indicating which patients with high-grade PIN have concomitant cancer.

The purinergic (P2X) receptors contribute significantly to Ca^{2+} influx to the cytoplasm, regulating at least 40 intracellular calcium-binding proteins. These control protein secretion, cell motility and adhesion, invasiveness, cytoskeletal modification, cell junction assembly, tissue differentiation, and phosphorylation as well as nuclear matrix protein composition and expression, cell cycle regulation and DNA transcription.[4] The ionotropic subtypes $P2X_1$ and $P2X_2$ are expressed in prostate epithelium when tumours are found, but not in normal tissue.[5,6] These changes occur simultaneously with the appearance of telomerase-associated protein.[6] The appearance of low levels of telomerase activity in apparently normal tissue adjacent to tumours, including lung, skin, gut, bladder, pancreas, kidney, cervix, vulva and prostate, is suggestive of a field-effect of biochemical changes associated with a biochemical transformation that is undetectable with the use of common histological stains.[7–9] It is unsurprising that several of the P2X receptors alter their expression in the acinar epithelial cells in the prostate in advance of the morphological changes associated with the development of prostate cancer given the major biochemical changes that occur in tissue undergoing neoplastic transformation.

The class of purinergic receptors of type P2X is comprised of fast, ligand-gated cation channels that open in response to the binding of extracellular ATP. The seven subtypes designated $P2X_{1-7}$ exhibit extensive homology (30–40%), particularly in structurally important elements such as the extracellular disulphide bridges. The long intracellular C-terminal domain unique to $P2X_7$ appears to confer time-dependent dilatation of the channel into a pore that possesses the capability of transporting large organic cations with molecular weights of up to several hundred Daltons that initiate apoptosis in the cell.[10–12]

Our aim has been to detect the early onset of prostate cancer in patients by identifying tissue either in the process of becoming neoplastic or that may be influenced by a tumour developing in adjacent prostatic tissue. A further aim has been to detect reliably the presence of existing, usually small low–moderate-grade tumours that may have escaped detection at biopsy sampling through the effects of the tumour on adjacent and morphologically normal prostatic epithelium and which are likely to become clinically significant.

Materials and methods

PROTOCOL DESIGN

Needle biopsies were tested from 116 men (age range 47–86 years) who had been biopsied more than once to detect the presence of prostate cancer. Twenty of the patients were biopsied three to four times, the remainder twice. Prostate samples, either needle biopsies or tissue obtained from transurethral resection, were also collected from 17 men (age 38–59 years) who had presented with symptoms of benign prostate hyperplasia between 5 and 10 years before confirmation that they remained free of prostate cancer. The earlier samples were tested from these men and six post mortem tissues were obtained from men aged 20–25 years for control purposes. These were fully sectioned at intervals to exclude cancer. No positive label was seen in associated tissue epithelium including urothelium. All samples were otherwise randomly selected. The 116 confirmed cancer cases were in the range Gleason sum score 4–9. Most of these patients were considered to be false negatives at the initial biopsy with tumours that were present but which were missed by the biopsy needles and were thus kept under close observation. They were consequently re-biopsied between 3 months and 6 years (up to three times) after the initial biopsies and were then all confirmed with cancer at a final biopsy when the tumour was directly sampled. Indications for re-biopsy included continuing high or increasing PSA levels and/or continued clinical suspicion based on ultrasound testing. Over half the cases were detected within 15 months of the initial biopsy. Sections (4–5 μm) were cut from an average of eight cores taken from the peripheral zone per procedure but ranging from three to 12 (apex, mid, base from left and right) for each sample.

TISSUE LABELLING

Each slide was de-waxed in two changes of fresh Histoclear for 10 min and then rehydrated. Sections were immersed in 0.3% hydrogen peroxide for 5 min, washed and then incubated for 30 min in affinity-purified human anti-$P2X_7$ antibody[3,12–15] at a concentration of 0.1 μg/ml IgG in Tris-buffered saline (TBS) at pH 7.2. Thereafter, slides were washed in TBS for 5 min, followed by 20 min incubation with 1 : 100 horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (DakoCytomation, Sydney, Australia). All slides were then washed in TBS for 5 min each, dried and mounted in Entellan mounting medium (Merck, Darmstadt, Germany). Approximately

serial sections of each block were stained with a standard haematoxylin and eosin (H–E) protocol. Peptide epitope (10 μM) completely blocked labelling in preadsorption controls while all other normal control sections were negative for $P2X_7$. The non-functional $P2X_7$ receptor antibody is scheduled to become commercially available by the end of 2004 as a monoclonal.

Results

NORMAL PROSTATE

Figure 1A shows a section of tissue stained by H–E from a prostate obtained from a young man established as being genuinely free of any prostate cancer. The diagnosis was made as a result of the continued absence of disease after a 5-year period and of the fact that the tissue was obtained from a man aged 40 presenting with urinary symptoms unrelated to prostate cancer. The serial section stained with anti-$P2X_7$ antibody in Figure 1B shows a total absence of stain indicating no up-regulated cytolytic $P2X_7$ receptor expression and thus no sign of early neoplasia, confirming the original diagnosis that the tissue was normal. Post mortem tissue obtained from young men (age 20–25) who were all free of prostate cancer all showed a similar complete lack of $P2X_7$ receptors in the prostate epithelium ($n = 6$). Similarly, normal breast[3] and skin epithelia are essentially devoid of $P2X_7$ receptors, while melanoma tissue appears heavily stained throughout the lesion.[16] Thus a total of 23 confirmed normal prostate tissues, all devoid of PIN and each sampled over wide regions of the tissue, were found to be devoid of all $P2X_7$ receptors in the acinar epithelial cells. Examples of this total lack of epithelial label for $P2X_7$ receptors are shown in Figure 1C–F.

CONFIRMED PROSTATE CANCER

The contrast with the appearance of the prostate epithelial cells from the 116 confirmed cancer cases was stark. Figure 2A,B shows two serial sections from a typical core through a moderate-grade tumour (Gleason sum score 6) with H–E stain (Figure 2A) and anti-$P2X_7$ antibody stain (Figure 2B). Ubiquitous labelling of $P2X_7$ receptors was found throughout all epithelial cells in the tumour tissue. The direct staining of tumours (Gleason sum scores 4–9) with the antibody to non-functional $P2X_7$ showed that 116/116 of the confirmed tumours exhibited a high density of cytolytic $P2X_7$ receptors in the affected epithelial cells. The receptors were generally condensed onto the apical membrane of the epithelial cells in higher-grade tumours (Gleason sum score 7–9) leaving little intracellular protein. Only when inserted in the cell membrane are $P2X_7$ receptors able to induce apoptosis. No tumour sample of any grade was unlabelled for $P2X_7$. An example is shown in Figure 3. A total of eight cores were collected from a patient identified with a localized moderate-grade tumour sampled in just two cores from the right lobe. Figure 3A,B shows regions of the two cores through tumour tissue in this patient that was labelled for $P2X_7$ receptor. Clear positive cytoplasmic and apical membrane labelling is apparent in the acini within both these cores. The other six cores showed no signs of cancer by H–E, yet each of these six ostensibly unaffected cores (Figure 3C–H) showed a dense concentration of intracellular cytoplasmic $P2X_7$. The protein generally was not expressed on the apical membrane. Each core collected from each of the 116 confirmed cancer cases exhibited distinct epithelial immunoreactivity for $P2X_7$. On average only 30% of the cores collected from each of the 116 patients directly sampled tumour tissue, the remainder sampling morphologically normal prostate tissue.

$P2X_7$ RECEPTOR EXPRESSION IN EARLY CANCER

The changing location and density of $P2X_7$ receptors in prostate epithelium was followed by examining all the biopsies collected prior to the final confirmation of cancer in the 116 confirmed cancer cases. Many of these cases, particularly those collected just 3–6 months before final positive identification of the cancer, were considered false negatives, as the tumour that must have been present at the earlier biopsy was simply missed in the process of sampling the tissue, a common occurrence with small confined tumours. All these cases displayed a characteristic appearance identical to that shown in Figure 3C–H. A distinct universal cytoplasmic intracellular distribution of $P2X_7$ receptors was found in all the epithelial cells even though overall cellular morphology appeared entirely normal by H–E.

An even earlier appearance of $P2X_7$ receptors in prostate epithelial cells was found in tissue biopsied between several months and 6 years prior to the positive confirmation of prostate cancer in the cohort of 116 patients. Some of these patients had exhibited PIN at initial biopsy. These much earlier samples showed apparently normal morphology in all sampled areas, with no sign of tumour in any core. An example is shown in the H–E-stained section in Figure 4A. However, staining for $P2X_7$ receptors in a serial section (Figure 4B) clearly showed that essentially all the

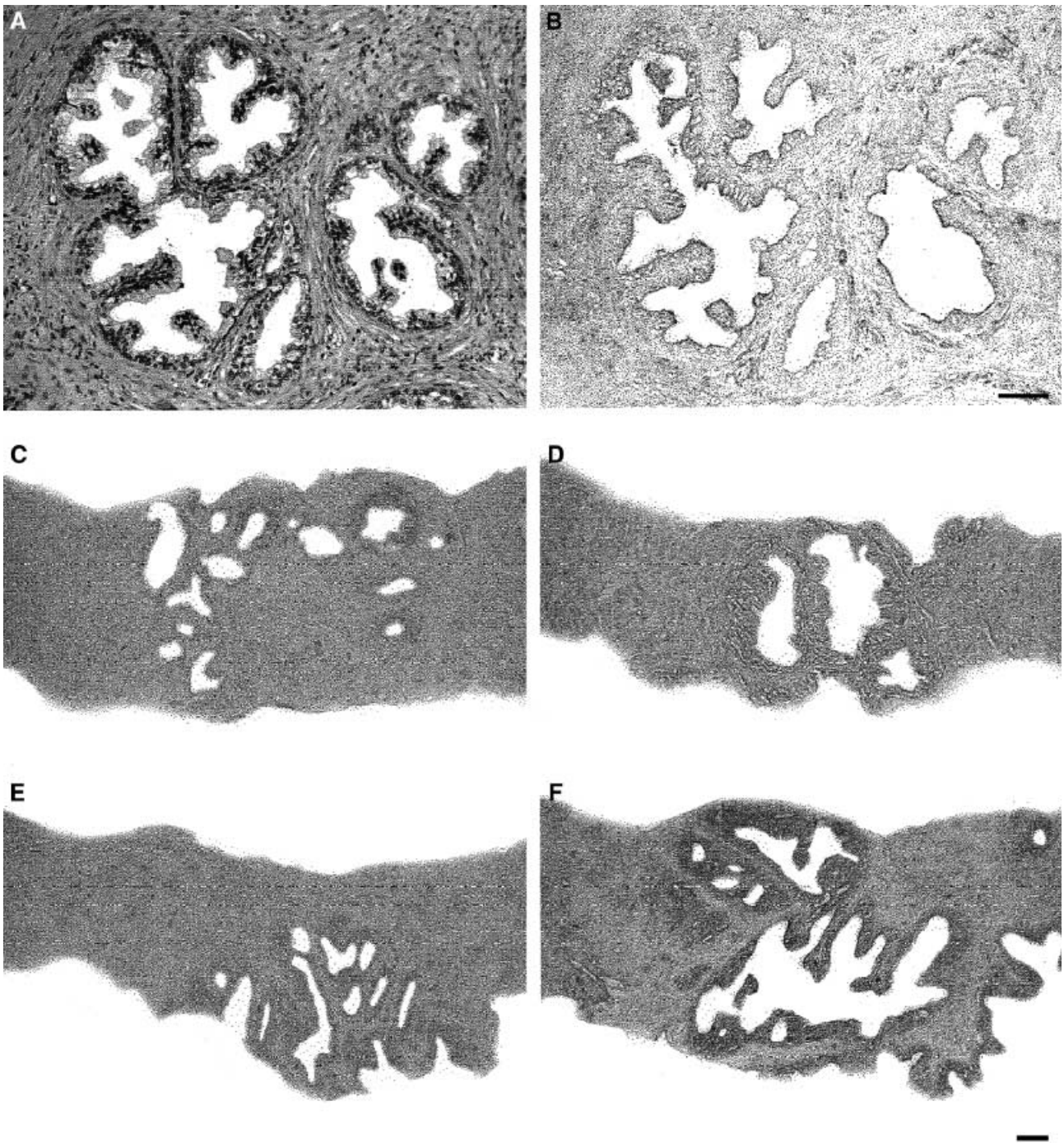

Figure 1. A,B, Serial sections of a prostate core from a patient with no early neoplasia or tumour and who was confirmed to be tumour free for 5 years after this biopsy was collected. **A,** H–E stain. **B,** The corresponding serial section is devoid of $P2X_7$ label. Identical results were obtained in normal tissue from young patients obtained at post mortem. **C–F,** Sections from four different cores collected from a patient subsequently found to be free of prostate cancer. All cores are unlabelled for $P2X_7$. Bars are 50 μm.

epithelial cell nuclei had begun to express $P2X_7$ (see arrows). Figure 4C–F shows areas from four cores obtained from a patient with no sign of cancer at the time of biopsy but who was diagnosed with cancer at a second biopsy investigation 3 years later. Clear nuclear $P2X_7$ label was found in every one of the initial set of six cores taken throughout this prostate. The appearance of $P2X_7$ protein in the epithelial cell nuclei

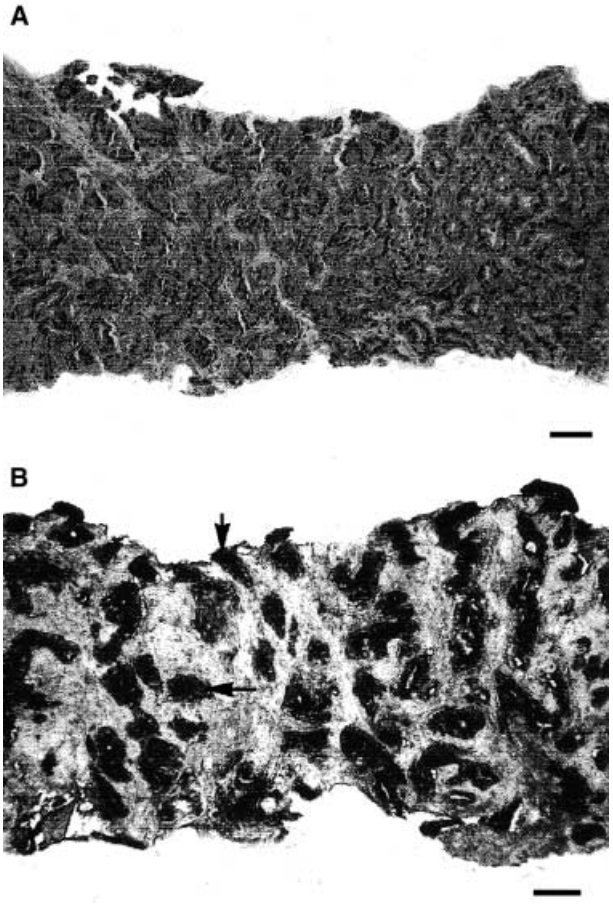

Figure 2. A,B, A core from a patient with moderate cancer (Gleason grade 6). The corresponding $P2X_7$ label shows that all receptors tend to condense on the apical membrane (arrow) of the acinar epithelial cells from within the cytoplasm. Every prostate tumour directly sampled in the core biopsies ($n = 116$) showed an identical pattern of staining in the epithelial cells, comparing starkly with all normal tissue that was all devoid of stain. Bars are 50 μm.

in these prostates appeared to be widespread from a very early stage of developing prostate cancer, before any tumour was detectable by H–E. There were very few acini that did not show $P2X_7$ expression by all luminal epithelial cell nuclei. Intermediate cases were found in which nuclear and cytoplasmic label was coextensive. Of the 116 patients confirmed with cancer, 38 exhibited nuclear receptor expression in tissue collected at earlier biopsies where no trace of cancer was found. They fell into two distinct groups. A total of 17 (44%) were confirmed with cancer by H–E analysis an average of 9 months later (range 6–15 months), while the remaining 21 (56%) were confirmed with cancer an average of 4 years later (range 2–6 years). The transformation rate between nuclear stain and morphologically distinct cancer was an indication of how rapidly the tumour developed. In rapidly developing cancers, the nuclear stain recorded at initial biopsy was replaced with cytoplasmic and apical membrane stain at the later biopsy in as short a time as several months. Several clinical indications such as rapid rise in PSA and/or suspicious digital rectal examination (DRE) alerted the clinicians to the need to quickly re-biopsy in these cases. Many other cases took several years for the prostatic luminal epithelium to undergo the transition in receptor distribution from nucleus to membrane.

This field effect showed that $P2X_7$ protein appeared in the nuclei throughout the prostate epithelium at least several months and up to 6 years before cancer was positively identified by H–E-stained biopsy. Moreover, this same field effect showed that intracellular cytoplasmic $P2X_7$ protein was always present in apparently normal tissue located well away from the site of the confirmed tumour. Tissue sections obtained from radical prostatectomies similarly showed widespread $P2X_7$ cytoplasmic staining throughout the entire sections and not just the sections through the tumour. There were no particular zonal differences found.

Discussion

The current study further validates earlier work using $P2X_1$ and $P2X_2$ to identify patients at risk of developing prostate cancer.[5,6] The study supports the suggestion that major changes in calcium regulatory mechanisms anticipate morphological changes associated with developing moderate- to high-grade neoplasia over times that range from as little as 6 months to 6 years. The faster the tumour grew the faster was the transition time between the appearance of nuclear protein label and the spread of the receptors to the cytoplasm and luminal epithelial cell membrane. $P2X_7$ cytolytic receptors were not found in any normal prostate epithelium in any section taken from 23 normal prostates, whereas the receptors were found in every section examined from 116 confirmed prostate cancer cases (Gleason sum scores 4–9); over 600 biopsied sections in all. In the highest grade prostate cancers (Gleason sum scores 7–9), where sections were taken through the tumour tissue, the pattern of receptor staining showed a distribution largely confined to the apical membranes of the epithelial cells in each acinus, presumably in a failed attempt to induce apoptosis in the affected cancer cells. In more moderate grade cancer tissue (Gleason sum scores 4–6), many of the receptors were found to be intracellular with a dense cytoplasmic distribution in the affected epithelial

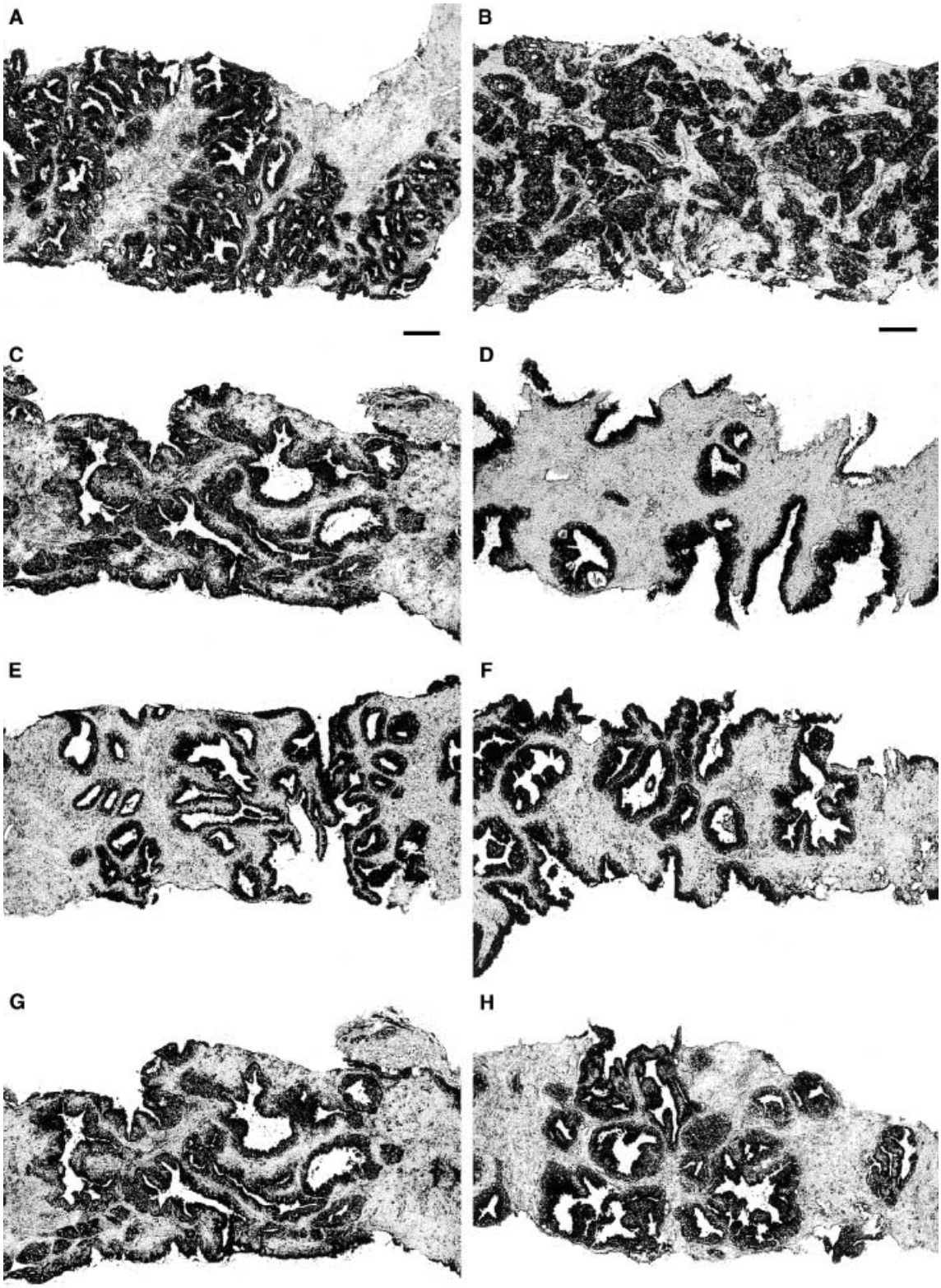

Figure 3. Sections from each of eight cores collected from a patient with prostate cancer and labelled with anti-$P2X_7$ antibody. **A,B,** Cores taken directly through the tumour. **C–H,** Sections from six different cores that missed the tumour. Each shows clear cytoplasmic epithelial cell label for $P2X_7$. All epithelial cells are labelled for $P2X_7$. Bars are 50 μm.

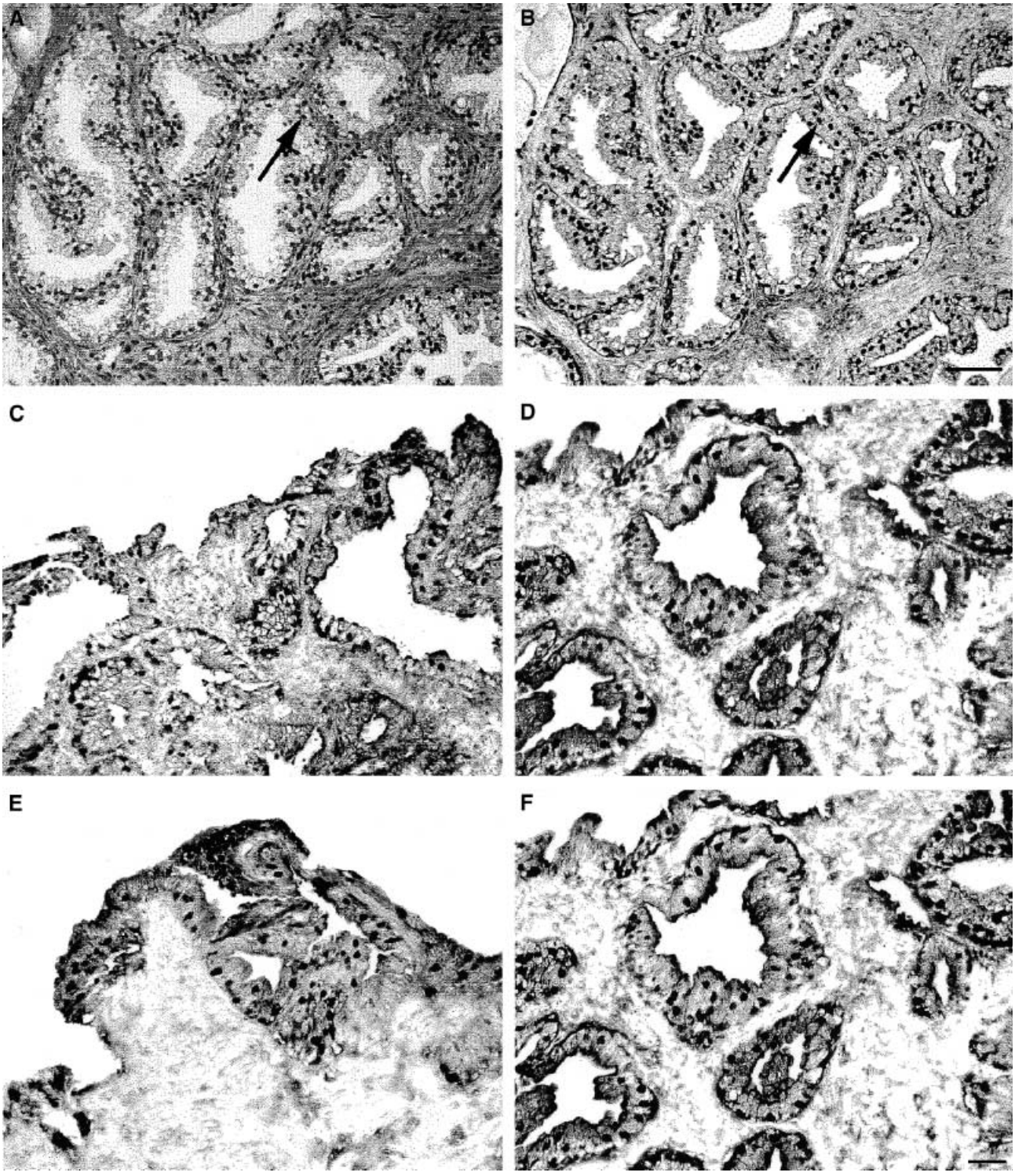

Figure 4. A,B, Serial sections of apparently normal prostate tissue showing a distinct pattern of $P2X_7$ receptor expression in each of the epithelial cell nuclei (arrows). All such cases ($n = 38$) developed clinically significant cancer within 6 years with nearly half displaying obvious tumour within 15 months. Bars are 50 μm. **C–F,** Sections from each of four cores labelled with anti-$P2X_7$ antibody collected from a patient 3 years before histological identification of a medium-grade contained prostate tumour was made. Distinct nuclear localization of the protein in the epithelial cells in all cores is apparent. Bars are 30 μm.

cells suggesting a high concentration of intracellular receptor not yet deployed on the cell surface. This same high intracellular concentration of receptors was found in apparently morphologically normal tissue well removed from the tumour margin, making detection of the tumour straightforward in those cases where the tumour may not have been sampled directly by the biopsy needle. No case of prostate cancer in this randomly selected cohort of 116 exhibited a different pattern of receptor distribution. Thus, no section of tissue collected from any confirmed tumour case displayed any acini that were not heavily labelled with anti-$P2X_7$ receptor antibody even in areas well removed from the identified tumour, including areas in the opposite lobe. The majority of this patient cohort have received or will soon receive major intervention for their cancer, including radical prostatectomy and radiotherapy. Thus most of the cancers detected at final biopsy were of the category requiring clinical intervention, either immediately or soon thereafter. All such cases requiring clinical intervention displayed receptor expression in epithelial cell cytoplasm and apical membrane. In contrast, very few unimportant or latent cancer conditions were selected in this cohort. Rather, these few cases fell into the category in which the receptor label apparently remained essentially nuclear in character.

This distinctly separate early stage of receptor distribution was seen in 38 of the 116 confirmed cases in the sets of biopsies collected at times that preceded tumour identification by several months to 6 years. No tissue that contained an identified clinically significant tumour exhibited this pattern of epithelial cell nuclear stain. Yet each of the 38 cases identified with $P2X_7$ confined to the epithelial cell nuclei developed into confirmed prostate cancer. The observed transition times between this early manifestation of receptors in the epithelial cell nuclei and detection of tumour fell into two distinct categories: an average of 9 months or an average of 4 years. These distinct categories suggest two broad categories of clinically significant tumour progression. Thus about half the observed tumours from this cohort appeared quite rapidly, while onset of the others was about five-fold slower. Because of the close monitoring of this cohort of patients very few of the tumours were first detected by needle biopsy with a Gleason sum score in excess of G6.

The pattern of receptor expression therefore appears capable of accurately identifying whether prostate cancer is absent (as defined here by an absence of $P2X_7$ receptors in the epithelial cells), is not yet clinically significant, may be classed as latent (as defined here by $P2X_7$ receptors confined within epithelial cell nuclei), or is already present in a clinically significant form (as indicated by the widespread appearance of intracellular $P2X_7$ receptors in the cytoplasm of the epithelial cells lining the acini). The presence of nuclear $P2X_7$ appears to be the initial response of cells to the need to boost the apoptotic mechanisms that are required to counter increased cell proliferation signals. An apoptotic mechanism operating through $P2X_7$ cytolytic receptors cannot occur until the protein is effectively assembled on the cell surface. However, the need for apoptotic pathways to be up-regulated in prostate tissue that detects altered proliferation signals is unsurprising. When this nuclear staining pattern was observed in tissue from a single biopsy it was also found in all other biopsies from the same patient, indicating that the up-regulation of $P2X_7$ in prostate epithelial cell nuclei is both rapid and widespread. Few samples were observed in which the nuclear stain was found alongside unstained acini.

The later stages of tumour development in which $P2X_7$ was found to have migrated into the cell cytoplasm and on to the plasma membrane were observed to occur much more slowly in regions remote from the actual tumour tissue. It was common to find regions in which both nuclear and cytoplasmic immunoreactivity were coextensive in very low-grade cancer cases (Gleason sum score 4), but this pattern was still present in epithelial cells that were remote from some much higher-grade tumours (Gleason sum score 7–8). The presence of every clinically significant tumour in the prostate, regardless of the Gleason grade, tumour size or the age of the patient, was characterized by clear cytoplasmic labelling for $P2X_7$ throughout the entire prostate epithelium ($n = 116$). Every tumour showed distinct cytoplasmic reactivity for $P2X_7$ in all the sampled acini irrespective of whether the acinus was a part of the tumour, was immediately adjacent to the tumour, or was in an otherwise normal region in the opposite lobe to the tumour. This is clearly seen in the example shown in Figure 3, in which a similar staining pattern is observed in all eight biopsy cores even though only two cores were taken through the tumour (Figure 3A,B). This method therefore appears to provide a reliable detection of the presence of clinically significant prostate tumours that are already present but not directly sampled with the biopsy needle, perhaps located in the difficult to reach apical lobe. The presence of the widespread cytoplasmic label for $P2X_7$ in biopsied tissue that appears morphologically normal seems to indicate the need either for an immediate set of more extensive repeat biopsies or else other continued close observation to detect the presence of the tumour that has gone unsampled.

Data obtained by $P2X_7$ immunolabelling appears to be unrelated to that of H–E staining other than in tissue from obvious tumour. Instead, the appearance of the potential cytolytic $P2X_7$ receptors flags underlying metabolic changes that precede the histological development of neoplasia well before the appearance of morphological changes associated with tumour development somewhere in the prostate, perhaps several centimetres from the affected epithelial cells. In contrast, recent work on superficial spreading melanoma ($n = 80$) has shown that anti-$P2X_7$ antibodies stain keratinocytes and melanocytes only in the affected tumour tissue with no field effect such as that evident in the prostate.[16] Like prostate, normal skin did not express the receptors. Thus, both skin and prostate cells up-regulate the expression of the apoptotic receptor in cancer cells without being able successfully to counteract increased cell proliferation.[16] The widespread labelling of telomerase-associated protein (hTP1) in the prostate epithelium in which a tumour was present[6] supports the observation that proliferation factors, perhaps including TP1,[6] act to overwhelm apoptosis. The $P2X_7$ apoptotic receptors become up-regulated in response to systemic and perhaps hormonal signals in the prostate to prepare for the onset of developing cancer. These apoptotic receptor proteins appear to be up-regulated throughout the prostate but are not deployed on the apical membrane of the epithelial cells except in those areas contained within the tumour. There is now clear evidence that these receptors are non-functional[15] in breast[3] as well as in skin[16] and prostate. The receptor deployment on the cell membrane is clearly unsuccessful, with the tumour gradually spreading and the affected epithelial cells unable to undergo apoptosis. This stage-related up-regulation of receptors in advance of the appearance of tumour indicates that the positive staining never indicates a false-positive result, with the process of neoplastic transformation taking several months to many years. No clinically significant tumour was detected by biopsy when the $P2X_7$ receptors were confined to the epithelial cell nuclei ($n = 38$), whereas no tissue in which a clinically significant tumour was found, however, localized, ever lacked cytoplasmic stain throughout both the tumour and adjacent tumour-free regions ($n = 116$).

Tumours that were rapidly developing could be differentiated from those that developed more slowly by observing the transition time between the appearance of $P2X_7$ receptors confined within the epithelial cell nuclei and receptors found throughout the cytoplasm. In this necessarily retrospective study, a rapid transition time (average 9 months) between nuclear and cytoplasmic receptor expression was associated with rapid tumour detection, while slower transition times (average 4 years) were associated with commensurately slower tumour detection times. The presence of a field effect underlying prostate cancer also is suggested by the strong genetic similarities seen between cancer and multifocal precursor lesions such as PIN.[17] These genetic similarities suggest that clonal expansion of precursor lesions such as PIN may account for the multifocal occurrence of prostate tumours.

Detection of the $P2X_7$ receptors does not depend on tumour type, Gleason grade, patient age or the presence or absence of preneoplastic conditions such as PIN. Moreover, patients found to be suffering from benign prostatic hyperplasia who do not show an up-regulation of $P2X_7$ receptors in the prostatic epithelium appear to be entirely free of any prostate cancer. These findings may provide new insights into mechanisms for controlling prostate cancer and may prove beneficial in monitoring those men who present with elevated serum PSA but in whom the biopsied tissue appears normal by H–E. The rate of progress of their disease may be followed by reference to the rate of transformation in $P2X_7$ receptor location. Ideally, this method should also be applied to prospective cohorts of patients followed over several years with the aim of separating patients with latent cancers not requiring clinical intervention (presumably those with steady-state nuclear label) from those cancers with cytoplasmic label that are clinically significant, requiring intervention.

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